INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR MUTANTS AND USES THEREOF

This invention was made with government support under Grants RO1-DK54568; RO1-DE14756 and PO1-DE13539 awarded by the National Institutes of Health. The government has certain rights in the invention.

I. BACKGROUND OF THE INVENTION

Inositol 1,4,5-trisphosphate receptors are intracellular ion channels that function to couple the activation of cell surface receptors for neurotransmitters, hormones, and growth factors to the initiation of intracellular Ca^{2+} release (1). Three genes have been cloned that encode distinct proteins of a molecular mass of ~300 kDa, named the type 1 ($InsP_3R$-1),1 type 2 ($InsP_3R$-2), and type 3 ($InsP_3R$-3) InsP3Rs (2-5). In addition, multiple receptor proteins with distinct tissue distributions are produced by alternate splicing of the type 1 receptor gene (7, 90). Notably, the type-1 receptor gene is alternatively spliced to yield additional variants of the receptor, which have specific tissue distribution [6, 7]. Most cells express multiple isoforms of $InsP_3R$ (91). Furthermore, the expression level and complement of receptors differ in individual tissues, and this together with regulation of the activity of the channel is thought to be a major determinant of the rich diversity of Ca^{2+} signaling events observed in cells (91, 12).

The functional channel is formed co-translationally by the tetrameric association of four individual receptor subunits (92, 93). Each subunit has a binding site for InsP3 toward the N terminus formed by a cluster of positively charged amino acids thought to coordinate the negatively charged phosphate groups of $InsP_3$ (8, 9, 94). The C terminus of each subunit is postulated to span intracellular membranes six times and forms a single cation-selective pore (10, 11, 95). In addition, this region signals retention of the protein to the endoplasmic reticulum (96, 97).

Although the $InsP_3$-binding pocket and channel pore are highly conserved between $InsP_3R$ family members, the intervening sequence between the binding region and pore is more divergent and consists of the so-called "regulatory and coupling" or "modulatory" domain. This region, consisting of ~1600 amino acids, is thought to be important in modulating the Ca^{2+} release properties of the $InsP_3R$. Indeed, Ca^{2+} release through the $InsP_3R$ is markedly influenced by many factors, most importantly by Ca^{2+} itself (98). $InsP_3R$ activity is also influenced through interaction with numerous factors such as proteins,

adenine nucleotides, and phosphorylation and in particular by cyclic nucleotide-dependent kinases (12).

This regulation of $InsP_3R$, together with the complement of $InsP_3R$ types and the sub-cellular localization of the channel, are thought to be the major determinants of the spatio-temporal characteristics of agonist-evoked Ca^{2+} signals [13, 14]. These particular characteristics likely contribute to the fidelity and specificity associated with activation of Ca^{2+}-dependent effectors. The most important regulator of $InsP_3$-induced Ca^{2+} release is Ca^{2+} itself [12, 15-17], however, numerous factors including adenine nucleotides [18, 19], protein interactions [12, 20] and phosphorylation by various kinases can significantly influence $InsP_3R$ function [7, 21-31]. In particular, since all $InsP_3R$ subtypes are phosphorylated by cAMP and cGMP-dependent protein kinases (PKA and PKG) [7, 21-23, 25, 26, 28, 29, 32-41], the $InsP_3R$ may represent an important nexus for cross-talk between these distinct signaling pathways. Indeed, cyclic nucleotide-dependent kinase-induced phosphorylation of $InsP_3R$ is proposed to be important in such diverse physiological and pathophysiological processes as synaptic plasticity [23], remodeling following neurotoxic insult [39], smooth muscle contractility [36, 37] and fluid secretion [29].

II. BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the invention and together with the description, serve to explain the principles of the invention.

Figure 1 shows the phosphorylation of S1755 results in enhanced Ca^{2+} release by $S2^+$ $InsP_3R$-1. In Figure 1A, DT40 3ko cells shown in the inset were transfected with M3 receptor, HcRed and $S2^+$ $InsP_3R$-1 as described below. Fura-2 loaded cells were stimulated with 50 nM Carbadol (CCh) to increase $[Ca^{2+}]_i$. A $[Ca^{2+}]_i$ increase was only evoked in cells expressing HcRed, and thus presumably M3 receptor and $S2^+$ $InsP_3R$-1 (compare black trace vs. gray trace from cells indicated in the inset). Treatment with 20 μM forskolin, to raise cAMP levels and activate PKA, resulted in a markedly enhanced CCh-induced $[Ca^{2+}]_i$ signal. After removal of forskolin a subsequent exposure to CCh resulted in a $[Ca^{2+}]_i$ increase similar to control. In Figure 1B, a similar experimental paradigm was utilized in DT-40 3ko cells expressing S1589A $S2^+$ $InsP_3R$-1. Stimulation with CCh following 20 μM forskolin also resulted in a markedly enhanced $[Ca^{2+}]_i$ increase relative to a control stimulation. Figure 1C shows that in cells expressing S1755A $S2^+$ $InsP_3R$-1, forskolin treatment did not result in an enhanced signal. Figure 1D shows pooled data from the

number of cells indicated for each construct, showing the normalized fold increase in initial $[Ca^{2+}]_i$ peak over control resulting from forskolin treatment of cells expressing $S2^+$ $InsP_3R$-1 and serine to alanine mutants. Forskolin treatment resulted in CCh responses in Wild type and S1589A $S2^+$ $InsP_3R$-1 cells being significantly different from S1755A $S2^+$ $InsP_3R$-1 expressing cells. Cartoon inset depicts the $S2^+$ $InsP_3R$-1 regulatory and coupling domain. The black shaded region represents the S2 splice region. The functionally important phosphorylation of S1755 is indicated by a gray circle.

Figure 2 shows the potentiation of Ca^{2+} release by forskolin after flash photolysis of ciInsP$_3$-PM. DT-40 3ko cells expressing $S2^+$ $InsP_3R$-1 were loaded with the visible wavelength Ca^{2+} indicator Fluo-4 and the cell permeable caged $InsP_3$ analog ciInsP$_3$-PM as described below. Figure 2A shows minimal photolysis of $InsP_3$ was evoked by a brief UV flash, (~0.5 msec indicated by the arrows) resulting in a small increase in $[Ca^{2+}]_i$. A subsequent, identical flash of UV light 5 min. later fails to evoke a larger increase in $[Ca^{2+}]_i$, although increasing the duration of the flash to 5 ms evokes a significantly larger increase in $[Ca^{2+}]_i$ (arrow, "max uncage"). In Figure 2B, an identical protocol was followed except the second minimal uncaging was performed following 5 min. treatment with 10 μM forskolin. This treatment resulted in a significantly larger increase in $[Ca^{2+}]_i$ when compared to the initial uncaging. Figure 2C shows pooled data comparing normalized fold increase for the first and second uncaging in the presence or absence of forskolin. Treatment with forskolin results in a statistically significant increase in the second response.

Figure 3 shows both S1589 and S1755 are functionally important PKA phosphorylation sites in $S2^-$ $InsP_3R$-1. A similar experimental paradigm as described in fig. 1 was utilized to assess the consequences and functionally important phosphorylation sites in $S2^-$ $InsP_3R$-1. Figure 3A shows treatment of wild type $S2^-$ $InsP_3R$-1 with forskolin resulted in enhanced CCh stimulated Ca^{2+} release with respect to a control CCh stimulation. Figure 3B shows a similar potentiation was observed with S1589A $S2^-$ $InsP_3R$-1 expressing cells. Figure 3C shows a similar enhanced $[Ca^{2+}]_i$ signal was observed following forskolin treatment in S1755A $S2^-$ $InsP_3R$-1 expressing cells. Figure 3D shows that no effect of forskolin treatment was observed in double mutant S1589A/S1755A $S2^-$ $InsP_3R$-1 expressing cells. Figure 3E shows pooled data for the number of cells indicated for each construct. The filled bars indicate data for the particular construct obtained using the low affinity Ca^{2+} indicator Fura-2FF. Normalized fold increase is only significantly altered in the double mutant. Cartoon inset depicts the functionally important phosphorylation of S1589 and S1755, indicated by gray circles.

Figure 4 shows the phosphorylation of S1755 by PKG results in enhanced Ca^{2+} release by $S2^+$ $InsP_3R$-1. A similar experimental paradigm utilized for experiments presented in fig. 1 was performed to assess the effects and site(s) of phosphorylation by PKG on $S2^+$ $InsP_3R$-1. Figure 4A shows treatment with 10 μM 8-Br cGMP to specifically activate PKG results in a marked potentiation of CCh-evoked Ca^{2+} release when compared to control CCh stimulation in the absence of PKG activation. Figure 4B shows a similar potentiation of Ca^{2+} release following PKG activation was observed in cells expressing S1589A $S2^+$ $InsP_3R$-1. Figure 4C shows that PKG activation does not enhance Ca^{2+} release by S1755A $S2^+$ $InsP_3R$-1. Figure 4D shows that PKG does not inhibit CCh-induced Ca^{2+} release by S1755A $S2^+$ $InsP_3R$-1. Figure 4E shows pooled data for the number of cells indicated for each construct. Normalized fold increase by S1755A $S2^+$ $InsP_3R$-1 was significantly different from both wild-type and S1589A $S2^+$ $InsP_3R$-1. Cartoon inset depicts the functionally important phosphorylation of S1755, indicated by a gray circle.

Figure 5 shows that treatment with PKI inhibits forskolin but not 8-Br cGMP-induced potentiation. Cells expressing $S2^+$ $InsP_3R$-1 were treated for 30 mins with myr-PKI(14-22) prior to assessing the effects of activating PKA or PKG. Figure 5A shows that PKI treatment completely abolishes the forskolin-induced enhancement of Ca^{2+} release. Figure 5B shows that PKI does not affect the 8-Br cGMP-induced enhancement of Ca^{2+} release. Figure 5C shows the results of pooled data.

Figure 6 shows PKG activation is without effect on Ca^{2+} release by $S2^-$ $InsP_3R$-1. A similar experimental paradigm used in experiments depicted in fig. 1 was utilized to assess the effects of PKG phosphorylation of $S2^-$ $InsP_3R$-1. Figure 6A shows that PKG activation has no effect on CCh-evoked Ca^{2+} release by wild-type $S2^-$ $InsP_3R$-1. Figure 6B shows that similarly no effect was observed in S1589A $S2^-$ $InsP_3R$-1 expressing cells. Figure 6C shows that no effect was observed in S1755A $S2^-$ $InsP_3R$-1 expressing cells. Figure 6D shows pooled data for the number of cells indicated for each construct. Cartoon depicts absence of phosphorylation by PKG.

Figure 7 shows in Figure 7A that ATP treatment of fura-2 loaded mouse parotid acinar cells, in the presence of la^{3+} to block Ca^{2+} entry (isolating P2Y receptors) results in an increase in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ is markedly enhanced by incubation with forskolin. Figure 7B shows that CCh treatment of human parotid acinar cells results in an increase in $[Ca^{2+}]_i$ which is potentiated by forskolin treatment. Representative traces from >4 experiments and >3 preparations of tissue.

Figure 8A depicts representative traces showing that mutation of both functional phosphorylation sites in the short form of $InsP_3R$-1 to glutamate residues results in a receptor which is apparently more sensitive to $InsP_3$ as revealed by increased sensitivity to CCh. Figure 8B shows the pooled data illustrating that the S1589E/S1755E mutant $InsP_3R$-1is approximately 7.5 fold more sensitive than the wild type S2- $InsP_3R$-1 and approximately 35 fold more sensitive than the S1589A/S1755A nonphosphorylatable S2-$InsP_3R$-1

Figure 9 shows single channel records from an isolated Cos-7 cell nucleus patched on several occasions. K^+ is the charge carrier, holding potential was +20 mV. Channel activity is observed only when $InsP_3$ is present in the pipette. Note in the final trace several channels appear to be present. Representative of 7 experiments.

Figure 10 shows a cartoon depicting the proposed structure of the $InsP_3R$.

Figure 11 depicts the regulatory and coupling domain of the $InsP_3R$-1; showing the phosphorylation sites at S1589 and S1755. In addition the location of the S2 splice site is shown.

Figure 12A shows that single mutation of S1589E in S2+ $InsP_3R$ has little effect on the potentiation of Ca^{2+} signaling seen upon phosphorylation of the receptor, confirming in the S2+ variant of the receptor that this site is not functional. Figure 12B/C show that stimulation of cells expressing the S1755E phosphomimetic mutation are apparently more sensitive to stimulation, and in additon no further enhancement of Ca^{2+} signaling is observed following PKA activation, confirming S1755 as the functionally important site.

Figure 13A/B shows that if either S1589 or S1755 is mutated to glutamate individually that no further potentiation by PKA stimulation is observed. Indicating that phosphorylation of individual sites is not functionally additive.

Figure 14 shows that $InsP_3R$-III can be phosphorylated in a PKA dependent fashion.

Figure 15A shows stimulation of DT40 cells expressing chicken $InsP_3$ R-III results in Ca^{2+} oscillations. Figure 15B shows that activation of PKA during these oscillations results in an inhibition of the Ca^{2+} signal, consistent with an effect on the $InsP_3R$-III.

Figure 16 shows that stimulation of DT-40 3ko transfected with rat $InsP_3R$-III results in Ca^{2+} signals which are inhibted by stimulating PKA.

Figure 17 shows flash photolysis of caged $InsP_3$ reveals that phosphomimetic $InsP_3R$-1 exhibits enhanced sensitivity to $InsP_3$. Figure 17A represents a schematic of amino acids 1586-1755 of the S2+ $InsP_3R$-1 showing the S2 splice site and potential PKA/PKG phosphorylation sites. Dt-40 3ko cells were transfected with either phosphomimetic

mutations of InsP3R-1 (B and D) or nonphosphorylatable mutations (C and E). Experiments in B and C are representative traces from cells transfected with the S2- $InsP_3R$-1 and in D and E with S2+ $InsP_3R$-1. The cells were loaded with the visible wavelength Ca^{2+} indicator fluo-4 and the cell-permeable caged $InsP_3R$ analogue ci-IP_3-PM as described under "Experimental Procedures." Increasing iIP_3 concentrations were generated by varying the duration (energy magnitude) of UV flash exposure from 0.5 to 5 mss. B, cells transfected with S2- EE constructs respond at a lower threshold of UV light exposure and with more robust elevations in $[Ca^{2+}]i$ than cells transfected with S2- AA mutations that are nonphosphorylatable (C). Similarly, as shown in D, S2+ SE mutations are more sensitive than S2+ SA mutations. The pooled data, expressed as fold increase in the $\Delta F/F_o$ fluorescence, are shown in F (S2- constructs) and G (S2+ constructs). The magnitude of responses to 1.25- and 2.5-ms exposure to UV light was significantly greater in phosphomimetic mutations in each splice variant, p 0.005. The values in parentheses indicate the number of cells responding above a 0.05% $\Delta F/F_o$ threshold in relation to the number of cells tested. For amino acid sequences shown, RRDS corresponds to SEQ ID NO: 25, RRES to SEQ ID NO: 26, RRDE to SEQ ID NO: 27, RREE to SEQ ID NO: 28, RREA to SEQ ID NO: 29.

Figure 18 shows the $InsP_3$ sensitivity of S2- $InsP_3R$-1 and phosphomimetic mutations. Concentration-response relationships for CCh-induced Ca^{2+} signals were examined in Dt-40 3ko cells expressing m3 receptors and $InsP_3R$-1 constructs. Figure 18A shows that cells transfected with S2- EE were stimulated with increasing concentrations of CCh as indicated (n = 9 cells). A similar paradigm was performed for cells expressing S2- WT (n = 10) (B) and for cells expressing S2- AA constructs (n = 9) (C). The magnitude of the initial peak (as an indicator of Ca^{2+} release) for each response was normalized to the maximum response in each cell. The pooled data and the fit that describes each concentration-response relationship for each construct is shown in D and illustrates that the sensitivity of Ca^{2+} release was greatest in S2- EE followed by S2- WT and S2- AA. For amino acid sequences shown, RRDE corresponds to SEQ ID NO: 27, RREE to SEQ ID NO: 28, RRDS to SEQ ID NO: 25, RRES to SEQ ID NO: 26, RRDA to SEQ ID NO: 30, and RREA to SEQ ID NO: 29.

Figure 19 shows the functional sensitivity of single phosphomimetic mutations in S2- $InsP_3R$-1. Figure 19A shows that concentration-response relationships were generated exactly as shown in Fig. 18D and as described under "Experimental Procedures." Cells expressing either S2- SE (n - 6) or S2- ES (n =5) displayed intermediate sensitivity between

S2- WT and S2- EE constructs. Dotted lines indicate S2- WT and S2- EE for comparison (data from Fig. 18D). Figure 19B shows that the threshold CCh-stimulated Ca^{2+} release is markedly potentiated by activation of PKA following forskolin treatment in cells expressing S2- WT. In contrast, no potentiation of threshold CCh-stimulated Ca^{2+} release is observed in cells expressing S2- SE in 19D, S2- ES in 19C, or S2- EE in 19E. Pooled data are shown in 19F; potentiation by forskolin is only seen in S2- WT. Numbers in parentheses indicate the number of analyzed cells. For amino acid sequences shown, RRDS corresponds to SEQ ID NO: 25, RRES to SEQ ID NO: 26, RRDE to SEQ ID NO: 27 and RREE to SEQ ID NO: 28.

Figure 20 shows the phosphorylation of S2- $InsP_3R$-1 mutants. The phosphorylation status of S2- InsP3R-1 constructs was assessed as described under "Materials and Methods." Figure 20A shows that incubation with 20 μM forskolin results in phosphorylation of Ser-1755 in S2- InsP3R-1. No phosphorylation is seen in S2- AA-transfected cells or untransfected HEK-293 cells (UN). Figure 20B shows that forskolin treatment results in phosphorylation of Ser- 1755 in S2- InsP3R-1 but not S2- EE, S2-ES, or S2- SE. The lack of phosphorylation of Ser-1755 in the S2- ES mutant is interpreted as indicating that mimicking phosphorylation of Ser-1589 by phosphomimetic substitution precludes the phosphorylation of Ser-1755 consistent with the functional data shown in Figure 19. IP, immunoprecipitation; WB, Western blot.

Figure 21 shows the functional sensitivity of S2+ $InsP_3R$-1. Concentration-response relationships were generated exactly as described previously. Figure 21A shows the normalized relationships for S2+ WT (n =11), S2+ SE (n =6), and S2+ SA (n =5) are shown. The fits illustrate the increased sensitivity of the S2+ SE relative to S2+ WT and S2+ SA constructs. Figure 21B shows the normalized concentration-response relationship for S2+ ES is shown with S2+ SE and S2+ WT (dotted lines for comparison). These data indicate that the sensitivity of S2+ ES is similar to S2+ WT.

Figure 22 shows the effects of PKA phosphorylation on S2+ $InsP_3R$-1 single glutamate substitution constructs. Threshold CCh-stimulated Ca^{2+} release is markedly potentiated by activation of PKA following forskolin treatment in S2+ WT-expressing cells (A) or S2+ ES (B). Figure 22C shows, in contrast, no potentiation of threshold CCh-stimulated Ca^{2+} release is observed in cells expressing S2+ SE. Figure 22D shows, pooled data illustrating that S2+ ES has properties identical to S2+ WT, and thus phosphorylation of this residue is unlikely to impact Ca^{2+} release. Numbers in parentheses indicate the number of analyzed cells.

Figure 23 shows the effect of PKG activation in single phosphomimetic constructs. Threshold CCh-stimulated Ca^{2+} release is markedly potentiated by activation of PKG following 8-Br-cGMP treatment in S2+ WT (A) or S2+ ES-expressing cells (B). Figure 23C shows, in contrast. no potentiation of threshold CCh-stimulated Ca^{2+} release is observed in S2+ SE-expressing cells. Figure 23D shows pooled data illustrating that S2+ ES has properties identical to S2+ WT, and thus phosphorylation of this residue following activation of PKG is unlikely to impact Ca^{2+} release. Numbers in parentheses indicate the number of analyzed cells.

Figure 24 shows a comparison of Ca^{2+} oscillations in Dt-40 3ko cells expressing S2- WT or phosphomimetic S2- constructs. Dt-40 3ko cells were transfected with either S2- WT (left panel) or S2- EE construct (middle panel). Figure 24A shows cells that were stimulated with a threshold concentration (250 ng/ml) of α-IgM antibody to stimulate the B cell receptor. In S2- WT-expressing cells, this resulted in a single Ca^{2+} transient after a long latency (A, left panel). In contrast, multiple Ca^{2+} transients were elicited in S2- EE-expressing cells following a shorter latency. Figure 24B shows cells that were stimulated with 500 ng/ml α-IgM antibody. Cells transfected with either S2- WT or S2- EE exhibited multiple Ca^{2+} transients of similar frequency, although the initial peak was generally larger and latency shorter in S2- EE-expressing cells. Figure 24C shows the pooled data from cells stimulated with various concentrations of α-IgM for frequency of oscillations is shown. Figure 24D is shown for latency, and 8E, is shown for the magnitude of the initial peak. Numbers in parentheses indicate the number of analyzed cells.

Figure 25 shows Fura-2 loaded DT-403ko cells transfected with M3R, Hc-red to identify transfected cells, and either wild-type (wt) (A) or PKA- phosphorylation site mutant $S2^-$ $InsP_3R$-I (B-D). Forskolin treatment results in potentiation of CCh responses in cells expressing wt, S1589A (SA) or S1755A (AS) mutants (A,B, and C respectively). Mutation of both putative PKA-phosphorylation sites results in loss of the potentiation (AA) (D).

Figure 26 shows Dt-403ko cells transfected with m3 receptor, $InsP_3R$-2 and HcRed (red trace) and untransfected cell (black trace). Incubation with forskolin (to activate PKA) but not 8Br-cGMP (to activate PKG) results in a marked potentiation of carbachol-induced Ca^{2+} signaling.

Figure 27A shows the sequence of the S2+ $InsP_3R$-1 and the region that is spliced out in the S2- $InsP_3R$-1 (shown in red). Splicing of the receptor creates a novel nucleotide binding motif (GXGXXG SEQ ID NO: 24). To disrupt the motif a glycine to alanine

mutation was constructed at amino acid 1690. As shown in Figure 27B in Dt-403ko cells expressing this construct, PKA activation fails to potentiate Ca^{2+} release signal induced by carbachol. For amino acid sequences shown, RRDS corresponds to SEQ ID NO: 25, RRES to SEQ ID NO: 26,

GYGEKQISIDELENAELPQPPEAENSTEELEPSPPLRQLEDHKRGEAL to SEQ ID NO: 31, GYGEKGEAL to SEQ ID NO: 32, and GXGXXG to SEQ ID NO: 24.

Figure 28 shows HEK-293 cells that were transfected with either wild type (WT) or nucleotide binding site mutant (G1690A) and treated with forskolin to activate PKA. The cells were subsequently lysed, proteins separated on SDS page and western blotted using an antibody which recognizes phosphorylated ser 1755 (top panel) or non-phosphorylated ser 1755 (bottom panel). The G1690A mutant is not phosphorylated following forskolin treatment.

III. DETAILED DESCRIPTION

The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the Examples included therein and to the Figures and their previous and following description.

Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that this invention is not limited to specific synthetic methods, specific recombinant biotechnology methods unless otherwise specified, or to particular reagents unless otherwise specified, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

Definitions

In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be

understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that when a value is disclosed that "less than or equal to" the value, "greater than or equal to the value" and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value "10" is disclosed the "less than or equal to 10"as well as "greater than or equal to 10" is also disclosed.

"Treatment" or "treating" means to administer a composition to a subject with an undesired condition or at risk for the condition. The condition can be any pathogenic disease, autoimmune disease, cancer or inflammatory condition. The effect of the administration of the composition to the subject can have the effect of but is not limited to reducing the symptoms of the condition, a reduction in the severity of the condition, or the complete ablation of the condition.

By "effective amount" is meant a therapeutic amount needed to achieve the desired result or results, e.g., increasing or decreasing Ca^{2+} release, enhancing or blunting physiological functions, altering the qualitative or quantitative nature of the proteins expressed by cell or tissues, and eliminating or reducing disease causing molecules and/or symptoms.

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

By "subject" is meant an individual. Preferably, the subject is a mammal such as a primate, and, more preferably, a human. The term "subject" can include domesticated animals, such as cats, dogs, etc., livestock (e.g., cattle, horses, pigs, sheep, goats, etc.), and laboratory animals (e.g., mouse, rabbit, rat, guinea pig, etc.).

By "$InsP_3R$" is meant an inositol 1,4,5-triphosphate receptor. Such receptors are generally the major route of intracellular calcium release in eukaryotic cells and are pivotal for stimulation of calcium dependent effectors. Modulation of calcium release through these receptors has important consequences in development and in a variety of normal and pathological cellular conditions. There are three major types of $InsP_3R$: $InsP_3R$-1, $InsP_3R$-2, and $InsP_3R$-3. $InsP_3R$-1 has two major splice variants: the $S2^-$ and the $S2^+$. The $S2^-$ splice

variant of $InsP_3R$-1 is the short splice variant in which 40 amino acids are excised. Specifically, residues 1693 to 1732 of the full length variant (ie. $S2^+$ or the long splice variant) are excised. The $S2^-$ variant is located predominantly in peripheral tissues whereas the $S2^+$ variant is present predominantly in the CNS.

By "homology" is meant the degree of relatedness shared between two or more nucleic acids, peptides, polypeptides or proteins as determined by their sequence structure or function.

It is understood that as discussed herein the use of the terms homology and identity mean the same thing as similarity. Thus, for example, if the use of the word homology is used between two non-natural sequences it is understood that this is not necessarily indicating an evolutionary relationship between these two sequences, but rather is looking at the similarity or relatedness between their nucleic acid sequences. Many of the methods for determining homology between two evolutionarily related molecules are routinely applied to any two or more nucleic acids or proteins for the purpose of measuring sequence similarity regardless of whether they are evolutionarily related or not.

In general, it is understood that one way to define any known variants and derivatives or those that might arise, of the disclosed genes and proteins herein, is through defining the variants and derivatives in terms of homology to specific known sequences. This identity of particular sequences disclosed herein is also discussed elsewhere herein. In general, variants of genes and proteins herein disclosed typically have at least, about 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to the stated sequence or the native sequence. Those of skill in the art readily understand how to determine the homology of two proteins or nucleic acids, such as genes. For example, the homology can be calculated after aligning the two sequences so that the homology is at its highest level.

Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Adv. Appl. Math. 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, J. MoL Biol. 48: 443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. U.S.A. 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment. It is understood that any of the methods typically can be used and that in certain instances the results of these various methods may differ, but the skilled artisan understands if identity is found with at least one of these methods, the sequences would be said to have the stated identity, and be disclosed herein.

For example, as used herein, a sequence recited as having a particular percent homology to another sequence refers to sequences that have the recited homology as calculated by any one or more of the calculation methods described above. For example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using the Zuker calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by any of the other calculation methods. As another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using both the Zuker calculation method and the Pearson and Lipman calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by the Smith and Waterman calculation method, the Needleman and Wunsch calculation method, the Jaeger calculation methods, or any of the other calculation methods. As yet another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using each of calculation methods (although, in practice, the different calculation methods will often result in different calculated homology percentages).

Mutants

The present invention provides mutant receptor proteins of two general categories: phosphomimetic mutant $InsP_3$ receptors and nonphosphorylatable mutant $InsP_3$. By "phosphomimetic" is meant a receptor that has an increased Ca^{2+} release function as compared to the wild-type $InsP_3R$ as a result of amino substitution to mimic phosphorylation. Preferably, the phosphomimetic mutant has a Ca^{2+} release function that is 3, 4, 5, 6, 7, 8, 9, 10 (or any amount in between) times that of the corresponding wild-type receptor. Preferably, the phosphomimetic mutant has a Ca^{2+} release function that is 10, 20, 30, 40 (or any amount in between) times that of the corresponding nonphosphorylatable

mutant. By "nonphosphorylatable" mutant is meant a "null" mutant that is not phosphorylated under conditions that cause phosphorylation in the wild-type. The mutants can be derived from $InsP_3R$-1, either the $S2^-$ or the $S2^+$ variant; $InsP_3R$-2; or $InsP_3R$-3.

By increased or enhanced Ca^{2+} release function is meant an increase release of calcium following a stimulus that activates the $InsP_3R$. Such stimuli include, for example, carbachol, an analog of acetylcholine, acting at muscarinic M3 receptors or alternatively, any agonist acting at any one of over one hundered plasma membrane receptors for neurotransmitters, hormones and growth factors coupled to the formation of $InsP_3$. In addition ehanced Ca^{2+} release can occur following direct activation of $InsP_3R$ with $InsP_3$ or its analogs.

The phosphomimetic mutants are derived by substitution of a serine in a phosphorylation site with a negatively charged amino acid residue. "By substitution of a serine in a phosphorylation site with a different amino acid residue" is meant that serine is removed and the different amino acid residue replaces it. By "a negatively charged amino acid residue" is meant that when incorporated into the protein it provides a net negative charge at the phosphorylation site. Thus, the substitution with the negatively charged amino acid residue neutralizes the positive charge at the site (provided at least by the typical arginine residue at the phosphorylation site). The phosphorylation site can be the strong PKA recognition motif of RXXS (SEQ ID NO:21), in which X represents any amino acid.. The serine residue can be replaced with either aspartate or glutamate. Thus the invention provides a $InsP_3R$ mutant comprising at least one substitution of serine with a negatively charged amino acid residue at a phosphorylation site of a wild-type $InsP_3R$, wherein the mutant has an enhanced Ca^{2+} release function as compared to the wild-type $InsP_3R$. Preferably the mutant's Ca^{2+} release function is at least 5 times greater than the Ca2+ release function of the wild-type $InsP_3R$. Preferably the mutant's Ca^{2+} release function is at least 10 times greater than the Ca^{2+} release function of the wild-type $InsP_3R$.

The invention provides a phosphomimetic $InsP_3R$-1 mutant, which has enhanced Ca^{2+} release function as compared to the wild-type $InsP_3R$-1. More specifically, the mutant comprises at least one substitution of serine with a negatively charged amino acid residue at a phosphorylation site, wherein the phosphorylation site is selected from residue 1589 or 1755 of a wild–type $InsP_3R$ sequence. As used throughout, the amino acid residues are numbered according to the rat sequences for the full length $InsP_3R$. Thus, one of skill in the art, can readily align the sequence for human (ATCC Acc. No. NM_002222), mouse

(ATCCAcc. No. NM_010585), or any other species and replace the comparable serine residue with the desired amino acid.

Examples of $InsP_3R$-1 phosphomimetic mutants include those wherein the substitution of serine with the negatively charged amino acid (including either glutamate or aspartate) is at residue 1589. Thus the mutant can comprise the amino acid sequence of SEQ ID NO:1, which corresponds to the short splice variant S1589E; SEQ ID NO:2, which corresponds to the long splice variant for S1589E; SEQ ID NO:3, which corresponds to S1589D in the short splice variant; SEQ ID NO:4, which corresponds to the long splice variant of S1589D.

The invention also provides the amino acids of SEQ ID NO:1, 2, 3,or 4 having one or more conservative amino acid substitutions, wherein the Ca^{2+} release function is maintained. Also provided are mutants comprising amino acid sequences having at least 80, 85, 90, 95, 96, 97, 98, 99 % (or any amount in between these values) homology to the amino acid sequence of SEQ ID NO:1, 2, 3, or 4 wherein the Ca^{2+} release function is maintained. Also provided are the comparable S1589E and S1589D mutants for various species.

Examples of $InsP_3R$-1 phosphomimetic mutants include those wherein the substitution of serine with the negatively charged amino acid (including either glutamate or aspartate) is at residue 1755. Thus the mutant can comprise the amino acid sequence of SEQ ID NO: 5; which corresponds to the short variant of S1755E; SEQ ID NO:6, which corresponds to the long splice variant of S1755E; SEQ ID NO:7, which corresponds to the short variant of S1755D; and SEQ ID NO:8, which corresponds to the long splice variant of S1755D.

The invention also provides the amino acids of SEQ ID NO: 5, 6, 7, and 8 having one or more conservative amino acid substitutions, wherein the Ca^{2+} release function is maintained. Also provided are mutants comprising amino acid sequences having at least 80, 85, 90, 95, 96, 97, 98, 99 % (or any amount in between these values) homology to the amino acid sequence of SEQ ID NO:5,6,7, or 8, wherein the Ca^{2+} release function is maintained. Also provided are the comparable S1755E and S1755D mutants for various species.

The invention also provides an $InsP_3R$-1 mutant, wherein the substitutions of serine with the negatively charged amino acid is at residues 1589 and 1755. Either glutamate or aspartate is substituted for the two serines, in any combination. Thus, the invention provides a mutant, wherein glutamate is substituted for serine at residues 1589 and 1755;

including for example a mutant comprising the amino acid sequence of SEQ ID NO:9, which corresponds to the S1589E/S1755E mutant of the short splice variant, or SEQ ID NO:10, which corresponds to the S1589E/S1755E mutant of the long splice variant. The invention also provides a mutant, wherein aspartate is substituted for serine at residues 1589 and 1755, including for example a mutant comprising SEQ ID NO:11, which corresponds to S1589D/S1755D of the short splice variant; SEQ ID NO:12, which corresponds to S1589D/S1755D of the long splice variant.

The invention also provides the amino acids of SEQ ID NO: 9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 having one or more conservative amino acid substitutions, wherein the Ca^{2+} release function is maintained. Also provided are mutants comprising amino acid sequences having at least 80, 85, 90, 95, 96, 97, 98, 99 % (or any amount in between these values) homology to the amino acid sequence of SEQ ID NO:9, 10, 11, or 12, wherein the Ca^{2+} release function is maintained. Also provided are the comparable S1589E/S1755E and S1589D/S1755D mutants for various species.

The invention further provides double mutants, wherein aspartate is substituted for serine at residue 1589 and glutamate is substituted for serine at residue 1755. For example, the invention provides a mutant comprising the amino acid sequence of SEQ ID NO:13, which corresponds to S1589D/S1755E short splice variant; and SEQ ID NO:14, which corresponds to a S1589D/S1755E mutation in the long splice variant. The invention also provides a mutant, wherein glutamate is substituted for serine at residue 1589 and aspartate is substituted for serine at residue 1755; including, for example, a mutant comprising the amino acid of SEQ ID NO: 15, which corresponds to a S1589E/S1755D mutant of the short splice variant; and a mutant comprising the amino acid of SEQ ID NO: 16, which corresponds to a S1589E/S1755D mutation in the long splice variant.

The invention also provides the amino acids of SEQ ID NO: 13, 14, 15, 16, having one or more conservative amino acid substitutions, wherein the Ca^{2+} release function is maintained. Also provided are mutants comprising amino acid sequences having at least 80, 85, 90, 95, 96, 97, 98, 99 % (or any amount in between these values) homology to the amino acid sequence of SEQ ID NO:13, 14, 15, or 16, wherein the Ca^{2+} release function is maintained. Also provided are the comparable S1589D/S1755E and S1589E/S1755D mutants for various species.

Similar to those outlined above for the $InsP_3R$-1 mutant, the invention provides phosphomimetic $InsP_3R$-2 mutants. More specifically, the mutant comprises at least one substitution of serine with a negatively charged amino acid residue at a phosphorylation

site, wherein the phosphorylation site is selected from residues 766, 1772, 1856, 1772, 1856, 2058, or 2227. For example, the sequence of SEQ ID NO: 19 can be modified to form S766E, S766D, S1772D, S1772E, S1856E, S1856D, S2058E, S2058D, S2227E, S2227D. Furthermore, any combination of these serine substations can be made to form mutants with two, three, four, or five different substitutions. As for the $InsP_3R$-1 mutants, the invention further provides substitution $InsP_3R$-1 mutants wherein the amino acid sequence modified from the wild type sequence, provided herein as SEQ ID NO:19, has one or more conservative amino acids, wherein the Ca^{2+} release function is maintained. Also provided are mutants comprising amino acid sequences having at least 80, 85, 90, 95, 96, 97, 98, 99 % (or any amount in between these values) homology to the amino acid sequence modified from the wild type sequence (provided herein as SEQ ID NO:19), wherein the Ca^{2+} release function is maintained. Also provided are the comparable $InsP_3R$-2 mutants for various species designed by aligning the $InsP_3R$-2 of the species with the rat species an substituting the corresponding amino acid sequence.

Similar to those outlined above for the $InsP_3R$-1 mutant, the invention provides phosphomimetic $InsP_3R$-3 mutants. More specifically, the mutant comprises at least one substitution of serine with a negatively charged amino acid residue at a phosphorylation site, wherein the phosphorylation site is selected from residues 934, 1640, 1834, 2009, 2041, or 2189. For example, the sequence of SEQ ID NO:20 can be modified to form S934E, S934D, S1640D, S1640E, S1834E, S1834D, S2009E, S2009D, S2041E, S2041D, S2189E, or S2189D. Furthermore, any combination of these serine substitutions can be made to form mutants with two, three, four, five, or six different substitutions. As for the $InsP_3R$-1 mutants, the invention further provides substitution $InsP_3R$-1 mutants wherein the amino acid sequence modified from the wild type sequence, provided herein as SEQ ID NO:20, has one or more conservative amino acids, wherein the Ca^{2+} release function is maintained. Also provided are mutants comprising amino acid sequences having at least 80, 85, 90, 95, 96, 97, 98, 99 % (or any amount in between these values) homology to the amino acid sequence modified from the wild type sequence (provided herein as SEQ ID NO:20), wherein the Ca^{2+} release function is maintained. Also provided are the comparable $InsP_3R$-2 mutants for various species designed by aligning the $InsP_3R$-2 of the species with the rat species and substituting the corresponding amino acid sequence.

The invention also provides mutants that are nonphosphorylatable. Specifically, the invention provides an $InsP_3R$ mutant comprising at least one substitution of serine with an amino acid with an aliphatic side chain at a phosphorylation site of a wild-type $InsP_3R$,

wherein the mutant is nonphosphorylatable. Preferably, the amino acid with the aliphatic side chain is alanine. The mutant can be a modified $InsP_3R$-1 (either the long or short splice variant), $InsP_3R$-2, or $InsP_3R$-3. Thus, the sequence of SEQ ID NO: 17 can be modified at residues 1589 and 1775 to form a null mutant, SEQ ID NO:18 can be modified at S1755 to form a null mutant. Similarly, nonphosphorylatable mutants of $InsP_3R$-2 can be formed by substituting alanine for any one or more of the residues 766, 1772, 1856, 2058, 2227 of a wild–type $InsP_3R$-2 sequence or any combination thereof. Nonphosphorylatable mutants of $InsP_3R$-3 can be formed by substituting alanine for any one or more of the residues 934, 1640, 1834, 2009, 2041, 2189 of a wild–type $InsP_3R$-3 sequence or any combination thereof. Similar mutants in various species can be derived by aligning the sequence with the rat sequence provided herein and making the null-inducing substitutions provided herein in the corresponding serine residue.

It is understood and herein contemplated that the short-form splice variants of an $InsP_3R$ comprise a unique binding motif GXGXXG (SEQ ID NO: 24) formed by the deletion of 40 amino acids. Thus, it is understood that the short form splice variant can be a variant of $InsP_3R$-1, $InsP_3R$-2, or $InsP_3R$-3. It is also understood and herein contemplated that the short-form splice variants formed by this deletion (e.g., the short form variant $InsP_3R$-1 S2- (SEQ ID NO: 17)) can comprise substitutions one or more of the amino acids that form the binding site. For example, one or more of the glycines at the unique binding site can be substituted with an alanine. Thus, for example, specifically contemplated are $InsP_3R$-1 S2- variant comprising a substitution of a glycine with an alanine at position 1690 (SEQ ID NO: 23). It is understood an herein contemplated that any of the glycines can be substituted with an alanine as described herein.

As discussed herein there are numerous variants of the $InsP_3R$ protein that are known and herein contemplated. In addition, to the known functional $InsP_3R$ strain variants, there are derivatives and fragments of the $InsP_3R$ proteins that also function in the disclosed methods and compositions. Protein variants and derivatives are well understood to those of skill in the art and can involve amino acid sequence modifications. For example, amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Immunogenic fusion protein derivatives, such as those described in the examples, are made by fusing a

polypeptide sufficiently large to confer immunogenicity to the target sequence by cross-linking *in vitro* or by recombinant cell culture transformed with DNA encoding the fusion. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the protein, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis. Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. The mutations must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 1 and are referred to as conservative substitutions.

TABLE 1:Amino Acid Substitutions

Original Residue	Exemplary Conservative Substitutions, others are known in the art.
Ala	Ser
Arg	Lys; Gln
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn, Lys
Glu	Asp
Gly	Pro
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln;
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe

Val; Ile; Leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 1, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the protein properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (e) by increasing the number of sites for sulfation and/or glycosylation.

For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative substitution. For example, a conservative substitution would be replacing one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are included within the mosaic polypeptides provided herein.

Substitutional or deletional mutagenesis can be employed to insert sites for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other labile residues also may be desirable. Deletions or substitutions of potential proteolysis sites, e.g. Arg, is accomplished for example by deleting one of the basic residues or substituting one by glutaminyl or histidyl residues.

Certain post-translational derivatizations are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and asparyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the o-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular

Properties, W. H. Freeman & Co., San Francisco pp 79-86 [1983]), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

It is understood that one way to define the variants and derivatives of the disclosed proteins herein is through defining the variants and derivatives in terms of homology/identity to specific known sequences. For example, SEQ ID NO: 1 sets forth a particular sequence of the "short form" ($S2^-$) of $InsP_3R$-1 (short form S1589E) and SEQ ID NO: 2 sets forth a particular sequence of the "long form" ($S2^+$) of $InsP_3R$-1 protein (long form S1589E). Specifically disclosed are variants of these and other proteins herein disclosed which have at least, 70% or 75% or 80% or 85% or 90% or 95% homology to the stated sequence. Those of skill in the art readily understand how to determine the homology of two proteins.

It is understood that the description of conservative mutations and homology can be combined together in any combination, such as embodiments that have at least 70% homology to a particular sequence wherein the variants are conservative mutations.

As this specification discusses various proteins and protein sequences it is understood that the nucleic acids that can encode those protein sequences are also disclosed. This would include all degenerate sequences related to a specific protein sequence, i.e. all nucleic acids having a sequence that encodes one particular protein sequence as well as all nucleic acids, including degenerate nucleic acids, encoding the disclosed variants and derivatives of the protein sequences. Thus, while each particular nucleic acid sequence may not be written out herein, it is understood that each and every sequence is in fact disclosed and described herein through the disclosed protein sequence. For example, one of the many nucleic acid sequences that can encode the protein sequence set forth in SEQ ID NO: 18 is set forth in SEQ ID NO: 22. In addition, for example, a disclosed conservative derivative of SEQ ID NO: 1 is shown in SEQ ID NO: 3, where the isoleucine (E) at position 1589 is changed to a valine (D). It is understood that for this mutation all of the nucleic acid sequences that encode this particular derivative of the short form S1589E are also disclosed. It is also understood that while no amino acid sequence indicates what particular DNA sequence encodes that protein within an organism, where particular variants of a disclosed protein are disclosed herein, the known nucleic acid sequence that encodes that protein is also known and herein disclosed and described.

Also provided are fragments of the proteins described below, wherein the fragments maintain the Ca^{2+} enhancing or reducing function of full length protein. Preferably the

fragment will have at least 50% of the enhancing function of the full length correlate or at least a 50% reduction of the Ca^{2+} reducing function.

Nucleic Acids, Vectors and Expression Systems

The invention further provides nucleic acids that encode the mutants described herein. Examples of nucleic acids that encode the $InsP_3R$-1 wild type receptors can be found at http://www.ncbi.nlm.nih.gov/HomoloGene/homol.cgi?HID=30927, including for example rat (ATCC Acc. No. xm_342732); mouse (ATCC Acc. No. nm_010585); human (ATCC Acc. No. nm_002222). Examples of nucleic acids that encode the $InsP_3R$-2 wild-type receptors can also be found at the http://www.ncbi.nlm.nih.gov site and include, for example, rat (ATCC Acc. No. NM_031046), human (ATCC Acc. No. NM_002223). Examples of nucleic acids that encode the $InsP_3R$-3 wild-type receptors can also be found at the http://www.ncbi.nlm.nih.gov site and include, for example, rat (ATCC Acc. No. NM_013138), human (ATCC Acc. No. NM_002224), and mouse (ATCC Acc. No. NM_080553).

One of skill in the art could modify these nucleic acid sequences to make the substitutions described herein. For example, the codon for the selected serine is replaced by a codon for glutamate, aspartate, or alanine. Further provided are nucleic acids that comprise a sequence that hybridizes under highly stringent conditions to the various rat, mouse, and human mutatnts encoding nucleic acids with the selected serine(s) substituted with glutamate, aspartate, or alanine. Preferably these hybridizing nucleic acids do not hybridize to the wild-type encoding nucleic acids.

The term hybridization typically means a sequence driven interaction between at least two nucleic acid molecules, such as a primer or a probe and a gene. Sequence driven interaction means an interaction that occurs between two nucleotides or nucleotide analogs or nucleotide derivatives in a nucleotide specific manner. For example, G interacting with C or A interacting with T are sequence driven interactions. Typically sequence driven interactions occur on the Watson-Crick face or Hoogsteen face of the nucleotide. The hybridization of two nucleic acids is affected by a number of conditions and parameters known to those of skill in the art. For example, the salt concentrations, pH, and temperature of the reaction all affect whether two nucleic acid molecules will hybridize.

Parameters for selective hybridization between two nucleic acid molecules are well known to those of skill in the art. For example, in some embodiments selective hybridization conditions can be defined as stringent or highly stringent hybridization conditions. For example, stringency of hybridization is controlled by both temperature and

salt concentration of either or both of the hybridization and washing steps. For example, the conditions of hybridization to achieve selective hybridization may involve hybridization in high ionic strength solution (6X SSC or 6X SSPE) at a temperature that is about 12-25°C below the Tm (the melting temperature at which half of the molecules dissociate from their hybridization partners) followed by washing at a combination of temperature and salt concentration chosen so that the washing temperature is about 5°C to 20°C below the Tm. The temperature and salt conditions are readily determined empirically in preliminary experiments in which samples of reference DNA immobilized on filters are hybridized to a labeled nucleic acid of interest and then washed under conditions of different stringencies. Hybridization temperatures are typically higher for DNA-RNA and RNA-RNA hybridizations. The conditions can be used as described above to achieve stringency, or as is known in the art. (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; Kunkel et al. Methods Enzymol. 1987:154:367, 1987 which is herein incorporated by reference for material at least related to hybridization of nucleic acids). A preferable stringent hybridization condition for a DNA:DNA hybridization can be at about 68°C (in aqueous solution) in 6X SSC or 6X SSPE followed by washing at 68°C. Stringency of hybridization and washing, if desired, can be reduced accordingly as the degree of complementarity desired is decreased, and further, depending upon the G-C or A-T richness of any area wherein variability is searched for. Likewise, stringency of hybridization and washing, if desired, can be increased accordingly as homology desired is increased, and further, depending upon the G-C or A-T richness of any area wherein high homology is desired, all as known in the art.

Another way to define selective hybridization is by looking at the amount (percentage) of one of the nucleic acids bound to the other nucleic acid. For example, in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the limiting nucleic acid is bound to the non-limiting nucleic acid. Typically, the non-limiting primer is in for example, 10 or 100 or 1000 fold excess. This type of assay can be performed at under conditions where both the limiting and non-limiting primer are for example, 10 fold or 100 fold or 1000 fold below their k_d, or where only one of the nucleic acid molecules is 10 fold or 100 fold or 1000 fold or where one or both nucleic acid molecules are above their k_d.

Another way to define selective hybridization is by looking at the percentage of primer that gets enzymatically manipulated under conditions where hybridization is required to promote the desired enzymatic manipulation. For example, in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer is enzymatically manipulated under conditions which promote the enzymatic manipulation, for example if the enzymatic manipulation is DNA extension, then selective hybridization conditions would be when at least about 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer molecules are extended. Preferred conditions also include those suggested by the manufacturer or indicated in the art as being appropriate for the enzyme performing the manipulation.

Just as with homology, it is understood that there are a variety of methods herein disclosed for determining the level of hybridization between two nucleic acid molecules. It is understood that these methods and conditions may provide different percentages of hybridization between two nucleic acid molecules, but unless otherwise indicated meeting the parameters of any of the methods would be sufficient. For example if 80% hybridization was required and as long as hybridization occurs within the required parameters in any one of these methods it is considered disclosed herein.

It is understood that those of skill in the art understand that if a composition or method meets any one of these criteria for determining hybridization either collectively or singly it is a composition or method that is disclosed herein.

Also provided are fragments of at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 nucleotides (or any number in between) of the nucleic acids provided herein, wherein the fragment encodes a serine substitution described herein.

The invention also provides an expression vector comprising a nucleic acid of the invention wherein the nucleic acid is operable linked to an expression control sequence.

The nucleic acids that are delivered to cells typically contain expression controlling systems. For example, the inserted genes in viral and retroviral systems usually contain promoters, and/or enhancers to help control the expression of the desired gene product. A promoter is generally a sequence or sequences of DNA that function when in a relatively fixed location in regard to the transcription start site. A promoter contains core elements required for basic interaction of RNA polymerase and transcription factors, and may contain upstream elements and response elements.

There are a number of compositions and methods which can be used to deliver nucleic acids to cells, either *in vitro* or *in vivo*. These methods and compositions can largely be broken down into two classes: viral based delivery systems and non-viral based delivery systems. For example, the nucleic acids can be delivered through a number of direct delivery systems such as, electroporation, lipofection, calcium phosphate precipitation, plasmids, viral vectors, viral nucleic acids, phage nucleic acids, phages, cosmids, or via transfer of genetic material in cells or carriers such as cationic liposomes. Appropriate means for transfection, including viral vectors, chemical transfectants, or physico-mechanical methods such as electroporation and direct diffusion of DNA, are described by, for example, Wolff, J. A., et al., *Science*, 247, 1465-1468, (1990); and Wolff, J. A. *Nature*, 352, 815-818, (1991). Such methods are well known in the art and readily adaptable for use with the compositions and methods described herein. In certain cases, the methods will be modifed to specifically function with large DNA molecules. Further, these methods can be used to target certain diseases and cell populations by using the targeting characteristics of the carrier.

Transfer vectors can be any nucleotide construction used to deliver genes into cells (e.g., a plasmid), or as part of a general strategy to deliver genes, e.g., as part of recombinant retrovirus or adenovirus (Ram et al. Cancer Res. 53:83-88, (1993)).

As used herein, plasmid or viral vectors are agents that transport the disclosed nucleic acids, such as SEQ ID NO: 22 into the cell without degradation and include a promoter yielding expression of the gene in the cells into which it is delivered. Viral vectors are, for example, Adenovirus, Adeno-associated virus, Herpes virus, Vaccinia virus, Polio virus, AIDS virus, neuronal trophic virus, Sindbis and other RNA viruses, including these viruses with the HIV backbone. Also preferred are any viral families which share the properties of these viruses which make them suitable for use as vectors. Retroviruses include Murine Maloney Leukemia virus, MMLV, and retroviruses that express the desirable properties of MMLV as a vector. Retroviral vectors are able to carry a larger genetic payload, i.e., a transgene or marker gene, than other viral vectors, and for this reason are a commonly used vector. However, they are not as useful in non-proliferating cells. Adenovirus vectors are relatively stable and easy to work with, have high titers, and can be delivered in aerosol formulation, and can transfect non-dividing cells. Pox viral vectors are large and have several sites for inserting genes, they are thermostable and can be stored at room temperature. A preferred embodiment is a viral vector which has been engineered so

as to suppress the immune response of the host organism, elicited by the viral antigens. Preferred vectors of this type will carry coding regions for Interleukin 8 or 10.

Viral vectors can have higher transaction (ability to introduce genes) abilities than chemical or physical methods to introduce genes into cells. Typically, viral vectors contain, nonstructural early genes, structural late genes, an RNA polymerase III transcript, inverted terminal repeats necessary for replication and encapsidation, and promoters to control the transcription and replication of the viral genome. When engineered as vectors, viruses typically have one or more of the early genes removed and a gene or gene/promotor cassette is inserted into the viral genome in place of the removed viral DNA. Constructs of this type can carry up to about 8 kb of foreign genetic material. The necessary functions of the removed early genes are typically supplied by cell lines which have been engineered to express the gene products of the early genes in trans.

A retrovirus is an animal virus belonging to the virus family of Retroviridae, including any types, subfamilies, genus, or tropisms. Retroviral vectors, in general, are described by Verma, I.M., Retroviral vectors for gene transfer. In Microbiology-1985, American Society for Microbiology, pp. 229-232, Washington, (1985), which is incorporated by reference herein. Examples of methods for using retroviral vectors for gene therapy are described in U.S. Patent Nos. 4,868,116 and 4,980,286; PCT applications WO 90/02806 and WO 89/07136; and Mulligan, (Science 260:926-932 (1993)); the teachings of which are incorporated herein by reference.

A retrovirus is essentially a package which has packed into it nucleic acid cargo. The nucleic acid cargo carries with it a packaging signal, which ensures that the replicated daughter molecules will be efficiently packaged within the package coat. In addition to the package signal, there are a number of molecules which are needed in cis, for the replication, and packaging of the replicated virus. Typically a retroviral genome, contains the gag, pol, and env genes which are involved in the making of the protein coat. It is the gag, pol, and env genes which are typically replaced by the foreign DNA that it is to be transferred to the target cell. Retrovirus vectors typically contain a packaging signal for incorporation into the package coat, a sequence which signals the start of the gag transcription unit, elements necessary for reverse transcription, including a primer binding site to bind the tRNA primer of reverse transcription, terminal repeat sequences that guide the switch of RNA strands during DNA synthesis, a purine rich sequence 5' to the 3' LTR that serve as the priming site for the synthesis of the second strand of DNA synthesis, and specific sequences near the ends of the LTRs that enable the insertion of the DNA state of the retrovirus to insert into

the host genome. The removal of the gag, pol, and env genes allows for about 8 kb of foreign sequence to be inserted into the viral genome, become reverse transcribed, and upon replication be packaged into a new retroviral particle. This amount of nucleic acid is sufficient for the delivery of a one to many genes depending on the size of each transcript. It is preferable to include either positive or negative selectable markers along with other genes in the insert.

Since the replication machinery and packaging proteins in most retroviral vectors have been removed (gag, pol, and env), the vectors are typically generated by placing them into a packaging cell line. A packaging cell line is a cell line which has been transfected or transformed with a retrovirus that contains the replication and packaging machinery, but lacks any packaging signal. When the vector carrying the DNA of choice is transfected into these cell lines, the vector containing the gene of interest is replicated and packaged into new retroviral particles, by the machinery provided in cis by the helper cell. The genomes for the machinery are not packaged because they lack the necessary signals.

The construction of replication-defective adenoviruses has been described (Berkner et al., *J. Virology* 61:1213-1220 (1987); Massie et al., Mol. Cell. Biol. 6:2872-2883 (1986); Haj-Ahmad et al., *J. Virology* 57:267-274 (1986); Davidson et al., *J. Virology* 61:1226-1239 (1987); Zhang, *BioTechniques* 15:868-872 (1993)). The benefit of the use of these viruses as vectors is that they are limited in the extent to which they can spread to other cell types, since they can replicate within an initial infected cell, but are unable to form new infectious viral particles. Recombinant adenoviruses have been shown to achieve high efficiency gene transfer after direct, *in vivo* delivery to airway epithelium, hepatocytes, vascular endothelium, CNS parenchyma and a number of other tissue sites (Morsy, *J. Clin. Invest.* 92:1580-1586 (1993); Kirshenbaum, *J. Clin. Invest.* 92:381-387 (1993); Roessler, *J. Clin. Invest.* 92:1085-1092 (1993); Moullier, *Nature Genetics* 4:154-159 (1993); La Salle, *Science* 259:988-990 (1993); Gomez-Foix, *J. Biol. Chem.* 267:25129-25134 (1992); Rich, *Human Gene Therapy* 4:461-476 (1993); Zabner, *Nature Genetics* 6:75-83 (1994); Guzman, *Circulation Research* 73:1201-1207 (1993); Bout, *Human Gene Therapy* 5:3-10 (1994); Zabner, *Cell* 75:207-216 (1993); Caillaud, *Eur. J. Neuroscience* 5:1287-1291 (1993); and Ragot, *J. Gen. Virology* 74:501-507 (1993)). Recombinant adenoviruses achieve gene transduction by binding to specific cell surface receptors, after which the virus is internalized by receptor-mediated endocytosis, in the same manner as wild type or replication-defective adenovirus (Chardonnet and Dales, *Virology* 40:462-477 (1970); Brown and Burlingham, *J. Virology* 12:386-396 (1973); Svensson and Persson, *J. Virology*

55:442-449 (1985); Seth, et al., *J. Virol.* 51:650-655 (1984); Seth, et al., *Mol. Cell. Biol.* 4:1528-1533 (1984); Varga et al., *J. Virology* 65:6061-6070 (1991); Wickham et al., *Cell* 73:309-319 (1993)).

A viral vector can be one based on an adenovirus which has had the E1 gene removed and these virons are generated in a cell line such as the human 293 cell line. In another preferred embodiment both the E1 and E3 genes are removed from the adenovirus genome.

Another type of viral vector is based on an adeno-associated virus (AAV). This defective parvovirus is a preferred vector because it can infect many cell types and is nonpathogenic to humans. AAV type vectors can transport about 4 to 5 kb and wild type AAV is known to stably insert into chromosome 19. Vectors which contain this site specific integration property are preferred. An especially preferred embodiment of this type of vector is the P4.1 C vector produced by Avigen, San Francisco, CA, which can contain the herpes simplex virus thymidine kinase gene, HSV-tk, and/or a marker gene, such as the gene encoding the green fluorescent protein, GFP.

In another type of AAV virus, the AAV contains a pair of inverted terminal repeats (ITRs) which flank at least one cassette containing a promoter which directs cell-specific expression operably linked to a heterologous gene. Heterologous in this context refers to any nucleotide sequence or gene which is not native to the AAV or B19 parvovirus.

Typically the AAV and B19 coding regions have been deleted, resulting in a safe, noncytotoxic vector. The AAV ITRs, or modifications thereof, confer infectivity and site-specific integration, but not cytotoxicity, and the promoter directs cell-specific expression. United States Patent No. 6,261,834 is herein incorproated by reference for material related to the AAV vector.

The vectors of the present invention thus provide DNA molecules which are capable of integration into a mammalian chromosome without substantial toxicity.

The inserted genes in viral and retroviral usually contain promoters, and/or enhancers to help control the expression of the desired gene product. A promoter is generally a sequence or sequences of DNA that function when in a relatively fixed location in regard to the transcription start site. A promoter contains core elements required for basic interaction of RNA polymerase and transcription factors, and may contain upstream elements and response elements.

Molecular genetic experiments with large human herpesviruses have provided a means whereby large heterologous DNA fragments can be cloned, propagated and

established in cells permissive for infection with herpesviruses (Sun et al., *Nature Genetics* 8: 33-41, 1994; Cotter and Robertson, *Curr Opin Mol Ther* 5: 633-644, 1999). These large DNA viruses (herpes simplex virus (HSV) and Epstein-Barr virus (EBV), have the potential to deliver fragments of human heterologous DNA > 150 kb to specific cells. EBV recombinants can maintain large pieces of DNA in the infected B-cells as episomal DNA. Individual clones carried human genomic inserts up to 330 kb appeared genetically stable The maintenance of these episomes requires a specific EBV nuclear protein, EBNA1, constitutively expressed during infection with EBV. Additionally, these vectors can be used for transfection, where large amounts of protein can be generated transiently *in vitro*. Herpesvirus amplicon systems are also being used to package pieces of DNA > 220 kb and to infect cells that can stably maintain DNA as episomes.

Other useful systems include, for example, replicating and host-restricted non-replicating vaccinia virus vectors.

The disclosed compositions can be delivered to the target cells in a variety of ways. For example, the compositions can be delivered through use of a gene gun, electroporation, or through lipofection, or through calcium phosphate precipitation. The delivery mechanism chosen will depend in part on the type of cell targeted and whether the delivery is occurring for example *in vivo* or *in vitro*.

Thus, the compositions can comprise, in addition to the disclosed vectors for example, lipids such as liposomes (cationic liposomes (e.g., DOTMA, DOPE, DC-cholesterol) or anionic liposomes). Liposomes can further comprise proteins to facilitate targeting a particular cell, if desired. Administration of a composition comprising a compound and a cationic liposome can be administered to the blood afferent to a target organ or inhaled into the respiratory tract to target cells of the respiratory tract. Regarding liposomes, see, e.g., Brigham et al. *Am. J. Resp. Cell. Mol. Biol.* 1:95-100 (1989); Felgner et al. *Proc. Natl. Acad. Sci USA* 84:7413-7417 (1987); U.S. Pat. No.4,897,355. Furthermore, the compound can be administered as a component of a microcapsule that can be targeted to specific cell types, such as macrophages, or where the diffusion of the compound or delivery of the compound from the microcapsule is designed for a specific rate or dosage.

In the methods described above which include the administration and uptake of exogenous DNA into the cells of a subject (i.e., gene transduction or transfection), delivery of the compositions to cells can be via a variety of mechanisms. As one example, delivery can be via a liposome, using commercially available liposome preparations such as LIPOFECTIN, LIPOFECTAMINE (GIBCO-BRL, Inc., Gaithersburg, MD), SUPERFECT

(Qiagen, Inc. Hilden, Germany) and TRANSFECTAM (Promega Biotec, Inc., Madison, WI), as well as other liposomes developed according to procedures standard in the art. In addition, the nucleic acid or vector of this invention can be delivered *in vivo* by electroporation, the technology for which is available from Genetronics, Inc. (San Diego, CA) as well as by means of a SONOPORATION machine (ImaRx Pharmaceutical Corp., Tucson, AZ).

The materials may be in solution, suspension (for example, incorporated into microparticles, liposomes, or cells). These may be targeted to a particular cell type via antibodies, receptors, or receptor ligands. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Senter, et al., *Bioconjugate Chem.*, 2:447-451, (1991); Bagshawe, K.D., *Br. J. Cancer*, 60:275-281, (1989); Bagshawe, et al., *Br. J. Cancer*, 58:700-703, (1988); Senter, et al., *Bioconjugate Chem.*, 4:3-9, (1993); Battelli, et al., *Cancer Immunol. Immunother.*, 35:421-425, (1992); Pietersz and McKenzie, *Immunolog. Reviews*, 129:57-80, (1992); and Roffler, et al., *Biochem. Pharmacol*, 42:2062-2065, (1991)). These techniques can be used for a variety of other speciifc cell types. Vehicles such as "stealth" and other antibody conjugated liposomes (including lipid mediated drug targeting to colonic carcinoma), receptor mediated targeting of DNA through cell specific ligands, lymphocyte directed tumor targeting, and highly specific therapeutic retroviral targeting of murine glioma cells *in vivo*. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Hughes et al., *Cancer Research*, 49:6214-6220, (1989); and Litzinger and Huang, *Biochimica et Biophysica Acta*, 1104:179-187, (1992)). In general, receptors are involved in pathways of endocytosis, either constitutive or ligand induced. These receptors cluster in clathrin-coated pits, enter the cell via clathrin-coated vesicles, pass through an acidified endosome in which the receptors are sorted, and then either recycle to the cell surface, become stored intracellularly, or are degraded in lysosomes. The internalization pathways serve a variety of functions, such as nutrient uptake, removal of activated proteins, clearance of macromolecules, opportunistic entry of viruses and toxins, dissociation and degradation of ligand, and receptor-level regulation. Many receptors follow more than one intracellular pathway, depending on the cell type, receptor concentration, type of ligand, ligand valency, and ligand concentration. Molecular and cellular mechanisms of receptor-mediated endocytosis has been reviewed (Brown and Greene, *DNA and Cell Biology* 10:6, 399-409 (1991)).

Nucleic acids that are delivered to cells which are to be integrated into the host cell genome, typically contain integration sequences. These sequences are often viral related sequences, particularly when viral based systems are used. These viral intergration systems can also be incorporated into nucleic acids which are to be delivered using a non-nucleic acid based system of deliver, such as a liposome, so that the nucleic acid contained in the delivery system can be come integrated into the host genome.

Other general techniques for integration into the host genome include, for example, systems designed to promote homologous recombination with the host genome. These systems typically rely on sequence flanking the nucleic acid to be expressed that has enough homology with a target sequence within the host cell genome that recombination between the vector nucleic acid and the target nucleic acid takes place, causing the delivered nucleic acid to be integrated into the host genome. These systems and the methods necessary to promote homologous recombination are known to those of skill in the art.

As described above, the compositions can be administered in a pharmaceutically acceptable carrier and can be delivered to the subject's cells *in vivo* and/or *ex vivo* by a variety of mechanisms well known in the art (e.g., uptake of naked DNA, liposome fusion, intramuscular injection of DNA via a gene gun, endocytosis and the like).

If *ex vivo* methods are employed, cells or tissues can be removed and maintained outside the body according to standard protocols well known in the art. The compositions can be introduced into the cells via any gene transfer mechanism, such as, for example, calcium phosphate mediated gene delivery, electroporation, microinjection or proteoliposomes. The transduced cells can then be infused (e.g., in a pharmaceutically acceptable carrier) or homotopically transplanted back into the subject per standard methods for the cell or tissue type. Standard methods are known for transplantation or infusion of various cells into a subject.

The invention also provides a cell comprising a vector described herein. Preferably the cultured cell, in the absence of the vector does not express $InsP_3Rs$. Optionally, the cell is a cultured cell. An example of such a cell is the DT-40 3ko cell. Optionally, the cell further comprises a nucleic acid that encodes an acetylcholine receptor (including for example an M3 receptor). Optionally various labels can be used to identify the cells that are successfully transfected or transformed.

Preferred promoters controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most

preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. beta actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication (Fiers et al., *Nature*, 273: 113 (1978)). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a *Hin*dIII E restriction fragment (Greenway, P.J. et al., *Gene* 18: 355-360 (1982)). Of course, promoters from the host cell or related species also are useful herein.

Enhancer generally refers to a sequence of DNA that functions at no fixed distance from the transcription start site and can be either 5' (Laimins, L. et al., *Proc. Natl. Acad. Sci.* 78: 993 (1981)) or 3' (Lusky, M.L., et al., *Mol. Cell Bio.* 3: 1108 (1983)) to the transcription unit. Furthermore, enhancers can be within an intron (Banerji, J.L. et al., *Cell* 33: 729 (1983)) as well as within the coding sequence itself (Osborne, T.F., et al., *Mol. Cell Bio.* 4: 1293 (1984)). They are usually between 10 and 300 bp in length, and they function in cis. Enhancers function to increase transcription from nearby promoters. Enhancers also often contain response elements that mediate the regulation of transcription. Promoters can also contain response elements that mediate the regulation of transcription. Enhancers often determine the regulation of expression of a gene. While many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, -fetoprotein and insulin), typically one will use an enhancer from a eukaryotic cell virus for general expression. Preferred examples are the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

The promotor and/or enhancer may be specifically activated either by light or specific chemical events which trigger their function. Systems can be regulated by reagents such as tetracycline and dexamethasone. There are also ways to enhance viral vector gene expression by exposure to irradiation, such as gamma irradiation, or alkylating chemotherapy drugs.

In certain embodiments the promoter and/or enhancer region can act as a constitutive promoter and/or enhancer to maximize expression of the region of the transcription unit to be transcribed. In certain constructs the promoter and/or enhancer region be active in all eukaryotic cell types, even if it is only expressed in a particular type of cell at a particular time. A preferred promoter of this type is the CMV promoter (650 bases). Other preferred promoters are SV40 promoters, cytomegalovirus (full length promoter), and retroviral vector LTF.

It has been shown that all specific regulatory elements can be cloned and used to construct expression vectors that are selectively expressed in specific cell types such as melanoma cells. The glial fibrillary acetic protein (GFAP) promoter has been used to selectively express genes in cells of glial origin.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells) may also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding tissue factor protein. The 3' untranslated regions also include transcription termination sites. It is preferred that the transcription unit also contain a polyadenylation region. One benefit of this region is that it increases the likelihood that the transcribed unit will be processed and transported like mRNA. The identification and use of polyadenylation signals in expression constructs is well established. It is preferred that homologous polyadenylation signals be used in the transgene constructs. In certain transcription units, the polyadenylation region is derived from the SV40 early polyadenylation signal and consists of about 400 bases. It is also preferred that the transcribed units contain other standard sequences alone or in combination with the above sequences improve expression from, or stability of, the construct.

The viral vectors can include nucleic acid sequence encoding a marker product. This marker product is used to determine if the gene has been delivered to the cell and once delivered is being expressed. Preferred marker genes are the *E. coli* lacZ gene, which encodes ß-galactosidase, and green fluorescent protein.

In some embodiments the marker may be a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase, neomycin, neomycin analog G418, hydromycin, and puromycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media. Two examples are: CHO DHFR- cells and mouse LTK- cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact

DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non-supplemented media.

The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, (Southern P. and Berg, P., *J. Molec. Appl. Genet.* 1: 327 (1982)), mycophenolic acid, (Mulligan, R.C. and Berg, P. *Science* 209: 1422 (1980)) or hygromycin, (Sugden, B. et al., *Mol. Cell. Biol.* 5: 410-413 (1985)). The three examples employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively. Others include the neomycin analog G418 and puramycin.

The invention further provides methods of using the mutants, nucleic acids, and cells of the invention in various methods of making the mutant receptors, methods of screening for agents that modulate the $InsP_3Rs$, and methods of treatment.

The expression systems described herein can be used to make mutant $InsP_3Rs$. Thus, the invention provides making mutant $InsP_3R$-1, $InsP_3R$-2, $InsP_3R$-3 or fragments thereof described herein by culturing a cell that expresses the selected mutant or fragments under conditions that allow expression and by isolating the expressed mutant or fragment thereof.

The invention further provides a method of screening for an agent that preferentially modulates Ca^{2+} release by phosphorylated $InsP_3R$, comprising contacting a cell of the invention with the agent to be screened, under conditions that allow Ca^{2+} release; measuring Ca^{2+} release; and comparing the amount of Ca^{2+} release with a control cell. The control cell can comprise an unphosphorylated $InsP_3R$ that is not contacted with the agent to be screened. Conditions that allow calcium release include for example carbachol, acting at muscaric M3 receptors or alternatively, an agonist acting at any one of over one hundered plasma membrane receptors for neurotransmitters, hormones and growth factors coupled to the formation of $InsP_3$. In addition, ehanced Ca^{2+} release can occur following direct activation of $InsP_3R$ with $InsP_3$ or its analogs. An increase or decrease in Ca^{2+} release as compared to a control cell indicates an agent that preferentially modulates unphosphorylated $InsP_3R$. By "modulation" is meant any physiologic effect that increases or decreases $InsP_3R$

stimulated calcium release. Preferably the unphosphorylated $InsP_3R$ is a nonphosphorylatable mutant $InsP_3R$ described herein.

The invention also provides a method of expressing a mutant InsP3R in a cell *in vivo*, comprising providing an expression vector described herein; introducing the vector into a cell *in vivo*; maintaining the cell under conditions that permit expression of the mutant $InsP_3R$ by the cell.

Also provided herein are methods of treating a subject with reduced Ca^{2+} release, comprising introducing into the subject an expression vector that encodes a phosphomimetic mutant of the invention, under conditions that an amount of the mutant receptor is expressed in an effective amount to enhance Ca^{2+} release. Examples of such diseases include, xerostomia, cystic fibrosis, or a large class of diseases which result from the decreased secretion of hormones, these include but are not limited to, decreased thyroid stimulating hormone (TSH) secretion from the pituitary or insulin secretion from the pancreas. The former deficiency results in dwarfism and cretinism, the latter diabetes.

Optionally the expression vector can be targeted. For example, to treat a subject with a condition like xerostomia, the vector could be targeted to the oral mucosal cells.

The invention also provides a method of treating a subject with cystic fibrosis, comprising introducing into the subject the expression vector of that expresses a phosphomimetic mutant receptor under conditions that an amount of the mutant receptor is expressed in an effective amount to alleviate the symptoms of cystic fibrosis.

It is understood and herein contemplated that the methods of treatment disclosed herein can also be used for the treatment of HIV and arthritis. Thus for example, specifically contemplated are methods of treating a subject with HIV comprising introducing into the subject the expression vector of the invention under conditions that an amount of $InsP_3R$ mutant is expressed in an effective amount to alleviate the symptoms of HIV. Also disclosed are methods of treating a subject with arthritis comprising introducing into the subject the expression vector of the invention under conditions that an amount of $InsP_3R$ mutant is expressed in an effective amount to alleviate the symptoms of arthritis.

Additional treatment methods are also contemplated. For example, the disclosed constructs can be used to inhibit apoptosis in a transplant. Thus, herein contemplated are methods of reducing apoptosis of cells of a transplant comprising introducing into the transplant a vector comprising one of the mutants disclosed herein. For example, the apoptosis of B cells being transplanted into a subject can be inhibited by introducing an $InsP_3R$ mutant into the B cells ex-vivo prior to administering the same cells to a subject. It

is understood and herein contemplated that "inhibit" or "inhibition" can refer to any treatment that results in any reduction of an event relative to an untreated condition. Thus, it is understood that inhibition can include a 5% reduction in the occurrence or presence of a condition or symptom as well as the complete ablation of an occurrence or presence of a condition or symptom. Thus, for example, inhibition of apoptosis can include a 5% reduction in the occurrence of cell death in a given cell population as well as the complete ablation of apoptosis related cell death in the same population.

EXAMPLES

Example 1: Phosphorylation of Type-1 Inositol 1,4,5-Trisphosphate Receptors by Cyclic Nucleotide-dependent Protein Kinases

Inositol 1,4,5-trisphosphate receptors ($InsP_3R$) constitute the major route of intracellular calcium release in eukaryotic cells and as such are pivotal for stimulation of Ca^{2+} dependent effectors important for numerous physiological processes. Modulation of this release has important consequences for defining the particular spatio-temporal characteristics of Ca^{2+} signals. In this study, regulation of Ca^{2+} release by phosphorylation of type-1 $InsP_3R$ ($InsP_3R$-1) by cAMP (PKA) and cGMP (PKG) dependent protein kinases was investigated in the two major splice variants of $InsP_3R$-1. $InsP_3R$-1 were expressed in DT-40 cells devoid of endogenous $InsP_3R$. In cells expressing the neuronal, $S2^+$ splice variant of the $InsP_3R$-1, Ca^{2+} release was markedly enhanced when either PKA or PKG was activated. The sites of phosphorylation were investigated by mutation of serine residues present in two canonical phosphorylation sites present in the protein. Potentiated Ca^{2+} release was abolished when serine 1755 was mutated to alanine (S1755A) but was unaffected by a similar mutation of serine 1589 (S1589A). These data demonstrate that S1755 is the functionally important residue for phosphoregulation by PKA and PKG in the neuronal variant of the $InsP_3R$-1. Activation of PKA also resulted in potentiated Ca^{2+} release in cells expressing the non-neuronal, $S2^-$ splice variant of the $InsP_3R$-1. However, the PKA-induced potentiation was still evident in S1589A or S1755A $InsP_3R$-1 mutants. The effect was abolished in the double (S1589A/S1755A) mutant, indicating both sites are phosphorylated and contribute to the functional effect. Activation of PKG had no effect on Ca^{2+} release in cells expressing the $S2^-$ variant of $InsP_3R$-1. Collectively these data indicated that phosphoregulation of $InsP_3R$-1 had dramatic effects on Ca^{2+} release and defined the molecular sites phosphorylated in the major variants expressed in neuronal and peripheral tissues.

Most studies of PKA-dependent phosphorylation have been performed on the $S2^+$ neuronal type-I $InsP_3R$, the so called "long-form" of the receptor. In this variant of the $InsP_3R$-1, serine residues at S1589 and S1755 are phosphorylated by PKA [7, 34, 35], with S1755 being more heavily phosphorylated [34]. In contrast, little consensus exists as to the effect of PKG; *in situ* experiments in cerebellar slices reported S1589 to be preferentially phosphorylated by PKG [35] , while other studies suggest that purified $InsP_3R$-1 protein from the cerebellum was phosphorylated preferentially on S1755 [36].

Alternative splicing of the type-1 receptor gene results in the $S2^-$ variant of the type-1 $InsP_3R$ where 40 amino acids (amino acids 1693 to 1732) are excised between the two phosphorylation sites [6, 7]. This protein is predominantly expressed in peripheral tissues and interestingly has been reported to be exclusively phosphorylated on S1589 by PKA [7], but on S1755 by PKG [36]. Studies of the functional effects of phosphorylation of the peripheral form have suggested that in contrast to the neuronal form of the receptor, phosphorylation of the "short form" of the $InsP_3R$-1 results in attenuated Ca^{2+} release [21, 22]. Thus, prior to this invention, the possibility existed that differences in both the sites of phosphorylation and therefore the functional effects of phosphorylation were defined by the particular splice variants expressed in particular tissues.

In this study the sites of phosphorylation by PKA and PKG, functionally important for regulation of Ca^{2+} release in the two major splice variants of the $InsP_3R$-1, were investigated. By expression of mutant $InsP_3R$-1 in $InsP_3R$ *null* DT-40 cells [42, 43], the studies revealed that phosphorylation of S1755 by PKA or PKG resulted in markedly enhanced Ca^{2+} release for $S2^+$ $InsP_3R$-1. Notably, in $S2^-$ $InsP_3R$ expressing cells PKG activation did not markedly alter Ca^{2+} release while PKA phosphorylation of both S1755 and S1589 result in enhanced Ca^{2+} release. Thus, the expression of particular $InsP_3R$ splice variants defined the functional consequences of phosphoregulation by cyclic nucleotide-dependent kinases.

Materials:

The acetoxymethylesters of Fura-2, and Fluo-4 were purchased from Molecular Probes (Eugene, Or.). Fura-2FF was purchased from Tefabs (Austin, TX). Cell permeable cyclic nucleotides and forskolin were purchased from Biomol, (Plymouth Meeting, PA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). The DT-40 cells lacking $InsP_3R$ (DT-40 3ko) were kindly provided by Dr Kurosaki, (Kansai Medical University, Japan) and were maintained as previously described [42-44].

Production of Mutations:

The $S2^+$ $InsP_3R$-1 in the expression plasmid pIRES-GFP (Clontech; Palo Alto, CA) was digested with the restriction endonuclease Sal I. The overhang created by digestion was blunted using T4 polymerase. An EcoR I linker was then ligated onto the blunted ends of the construct. The entire receptor DNA was excised from the plasmid using EcoR I and ligated into the plasmid MXT-1. The region containing the $S2^-$ splice variant and potential PKA phosphorylation sites was excised from its backbone in pCDNA 3.1+ (Invitrogen; Carlsbad, CA) by Rsr II and Kas I and ligated into the $InsP_3R$ construct in MXT-1. The potential PKA sites, S1589 and S1755, were mutated, individually in both splice variants and together in the $S2^-$ splice variant, to alanines using sequential PCR mutagenesis. The outer oligos used for the mutagenesis reaction flanked the restriction sites Rsr II and Kas I. Following mutation, the resulting fragments were cut with Rsr II and Kas I and inserted into the IP_3R-1 backbone at the corresponding sites. The mutations and lack of spurious misincorporations were confirmed by Big Dye fluorescent sequencing. Mutated receptor DNAs were excised from MXT-1 using EcoR I and ligated into the mammalian expression vector pGW (British Biotechnology; Oxford, UK). Orientation was confirmed using restriction enzyme digestion.

Transfection of DT-40 cells:

DT-40 cells lacking all three $InsP_3$ receptor subtypes DT-40 3kowere transfected using electroporation, 350 V and 950 μF. 2 x 10^7 cells were co-transfected with 25 μg of the $InsP_3R$ cDNA, 25 μg of the muscarinic type 3 (M3) receptor, and 4 μg of the red fluorescent protein plasmid pHcRed1-N1. Cells were incubated with DNA in 500 μl of Optimem media on ice for 10 minutes. The cell / DNA mixture was electroporated, incubated on ice for 30 minutes, brought up to 5 ml with Optimem and placed in a 5 % CO_2 incubator at 39 °C for 5 hours. The cells were then centrifuged and resuspended in 12 ml of complete RPMI media. Transfection efficiency was typically ~20%. Experiments were performed within 32 hours of transfection.

Digital Imaging of $[Ca^{+2}]_i$:

Transfected DT-40 3ko cells were washed once in a HEPES-buffered physiological saline solution (HEPES-PSS) containing (in mM) 5.5 glucose, 137 NaCl, 0.56 $MgCl_2$, 4.7 KCl, 1 Na_2HPO_4, 10 HEPES (pH 7.4), 1.2 $CaCl_2$ and 1% w/v Bovine Serum Albumin. Cells were then resuspended in BSA HEPES-PSS with 1 μM Fura-2 (AM), placed on a 15 mm glass coverslip in a low volume perfusion chamber (Warner Instruments) and allowed to adhere for 30 minutes at room temperature. Cells were perfused continually for 10 minutes

with HEPES-PSS before experimentation to allow Fura-2 de-esterification. A field of cells for each experiment was chosen that contained a wide range of transfection efficiency based upon the intensity of red fluorescence emitted when excited at 560 nm. $[Ca^{2+}]_i$ imaging was performed essentially as previously described [28, 29, 41] using an inverted epifluorescence Nikon microscope with a 40X oil immersion objective lens (numerical aperture, 1.3). Cells were excited alternately with light at 340 and 380 nm (± 10 nm bandpass filters, Chroma) using a monochrometer (TILL Photonics). Fluorescence images were captured and digitized with a digital camera driven by TILL Photonics software. Images were captured every 2 seconds with an exposure of 35 ms and no binning. 340 / 380 ratio images were calculated online and stored immediately to hard disk. Only data from cells exhibiting an increase in ratio units of less than 0.2 upon stimulation were used for further analysis.

Flash Photolysis:

Transfected cells were simultaneously loaded by incubation with the visible wavelength indicator Fluo-4 and a cell permeable form of caged-inositol trisphosphate (ciIP_3/PM) for 30 min. Ci-IP_3/PM is a homologue of cm-IP_3/PM [45] The 2- and 3-hydroxyls of the inositol ring were protected by an isopropylidene group in ci-IP3/PM, and were protected by a methoxymethylene group in cm-IP_3/PM. Like cm-IP3/PM, ci-IP3/PM diffuses across cell membranes and induces internal calcium release upon photo-uncaging. A further period of approximately 30 min was allowed for de-esterification of both dye and cage. Cells were illuminated at 488 ± 10 nm and fluorescence collected through a 525 ± 25 nm band pass filter and captured using the Till Photonics imaging suite. These traces are displayed as % $\Delta F/F_0$, where F is the recorded fluorescence and F_0 is the mean of the initial 10 sequential frames. Photolytic release was performed as previously described [28, 29, 41] using a pulsed Xenon arc lamp (Till Photonics). A high intensity (0.5-5 msec duration; 80 J) discharge of UV light (360 ± 7.5 nm) was reflected onto the plane of focus using a DM400 dichroic mirror and Nikon X 40 oil immersion objective, 1.3 NA.

Statistical Analysis:

The effects of treatment were determined by normalizing the peak change in fluorescence ratio by stimulation following forskolin or 8-Br cGMP exposure to that of stimulation in control HEPES-PSS. Thus, pooled data represents a normalized fold increase over control for the treated trial. Two tailed heteroscedastic t-tests with P values < 0.05 were considered to have statistical significance.

PKA phosphorylation of S1755 in $S2^+$ $InsP_3R$-1 resulted in enhanced Ca^{2+} release:

Experiments were performed in DT-40 3ko cells transfected with $S2^+$ $InsP_3R$-1. Since there are no reports of Gαq coupled receptors expressed in DT-40 cells, initial experiments were performed eliciting $[Ca^{2+}]_i$ changes by stimulating the endogenous B cell receptor with α-IgM antibody. This resulted in somewhat irregular Ca^{2+} oscillations, which were not reversible when the antibody was removed making paired analysis of any effects of raising cyclic nucleotides difficult to interpret. Thus, in all further experiments, DT-40 3ko cells were co-transfected with the M3 receptor, and HcRed to facilitate identification of transfected cells (inset fig. 1). Stimulation of M3 receptors with the muscarinic agonist carbachol (CCh), provided a convenient means of stimulating $[Ca^{2+}]_i$ changes, presumably through Gαq-induced activation of phospholipase C and production of $InsP_3$. Stimulation with a low concentration of CCh (25-50 nM) resulted in an increase in $[Ca^{2+}]_i$, which returned to baseline when the agonist was removed. Following 10 min incubation with 20 μM forskolin to maximally raise cAMP, the initial peak in $[Ca^{2+}]_i$ elicited by identical CCh treatment was markedly potentiated (fig 1A and pooled data in fig 1D). After washout of forskolin a subsequent stimulation resulted in a $[Ca^{2+}]_i$ change comparable to the initial exposure. The potentiation was most marked when threshold elevations in $[Ca^{2+}]_i$ were evoked by the initial exposure to CCh and therefore only cells in which the initial CCh treatment evoked a Δ 340/380 ratio of < 0.2 ratio units were included for analysis. These data showed that phosphorylation of the $S2^+$ $InsP_3R$ resulted in enhanced Ca^{2+} release by increasing the sensitivity of the receptor to $InsP_3$. $[Ca^{2+}]_i$ changes were never evoked by CCh treatment in cells transfected with only M3 cDNA in the absence of $InsP_3R$ or likewise $InsP_3R$ with no M3 cDNA. Similarly, cells exhibiting no HcRed fluorescence seldom responded to CCh treatment (black trace, fig 1A).

To ascertain whether one or both of the serine residues S1589 or S1755 is important for phosphoregulation of the $S2^+$ variant of the $InsP_3R$-1 following PKA activation, individual point mutations were constructed where these serine residues were mutated to alanine (S1589A and S1755A). A similar potentiation of the initial peak of the CCh-induced $[Ca^{2+}]_i$ elevation was observed following forskolin treatment in cells expressing S1589A $S2^+$ $InsP_3R$ (fig 1B and 1D), however mutation of S1755A resulted in the complete abrogation of any potentiation upon forskolin treatment (fig 1C and 1D). These data clearly support the assertion that phosphorylation of S1755 is the important event underlying enhanced Ca^{2+} release through the neuronal $InsP_3R$-1 following PKA activation. It follows

therefore, that phosphorylation of S1589 after 20 μM forskolin treatment (which could reasonably be expected to result in the maximal generation of cAMP) was either not occurring to a significant extent, or perhaps more likely, was functionally not important in modulating Ca^{2+} release.

Since the only difference between the experiment in fig 1A and 1C is a conservative point mutation in the $InsP_3R$-1, it was assumed that the effects observed on the peak Ca^{2+} signal following forskolin treatment were predominately the results of alteration of Ca^{2+} release. To rule out the possibility that phosphorylation by PKA of other signaling molecules caused the effect, experiments were performed utilizing $ciIP_3$/PM, a cell permeable form of caged $InsP_3$ [45] to more directly induce Ca^{2+} release in cells transfected with wild-type $S2^+$ $InsP_3R$-1. Using a brief flash of UV light (~ 0.5 ms, indicated by the arrow in fig 2) small elevations in $[Ca^{2+}]_i$ could be evoked. Subsequent exposure to UV light never produced an increase larger than that initially evoked, however longer flashes of UV light (5 msec) could evoke larger peak increases (fig 2A; max uncage, indicated by the large arrow-head). No effect of UV light was observed in cells either not loaded with cage, or alternatively not expressing $InsP_3R$. In contrast, when cells were incubated with 10 μM forskolin for 5 min prior to a second identical exposure to UV light a marked increase in the initial Ca^{2+} peak was evoked (fig 2B and pooled data fig 2C). This potentiation of $InsP_3$-induced release was of similar magnitude to that seen for CCh-treated cells exposed to forskolin and supports the notion that the predominant effect of forskolin treatment is to regulate Ca^{2+} release through phosphorylation of $InsP_3R$.

PKA-induced phosphorylation of S1589 and S1755 were functionally important in the $S2^-$ $InsP_3R$-1:

The $S2^-$ variant of the $InsP_3R$-1 is predominantly expressed in peripheral tissues and in fetal brain during neuronal development [6, 7]. The $S2^-$ $InsP_3R$-1 has been reported to be phosphorylated by PKA in a number of tissues, including platelets, vas deferens, smooth muscle and hepatocytes [7, 30, 46]. In contrast to the neuronal form of the $InsP_3R$-1, studies performed with $S2^-$ $InsP_3R$-1 purified from vas deferens and smooth muscle have demonstrated that the receptor is almost exclusively phosphorylated on S1589 [7]. Reports have also suggested a different functional outcome as a result of PKA activation, since the majority of studies, for example in megakaryocytes have suggested that phosphorylation results in inhibition of Ca^{2+} release [22]. An important caveat relevant to the interpretation of this functional data, is that in contrast to $InsP_3R$-1 in cerebellum (>99% $S2^+$ $InsP_3R$-1), in

peripheral tissues multiple $InsP_3R$ types are invariably expressed to varying degrees [47]. Thus, unequivocally attributing an effect to an individual homotetrameric receptor was problematic prior to the invention.

Similar experimental paradigms were employed to assess the effect of PKA-induced phosphorylation on a homogeneous population of $S2^-$ $InsP_3R$-1. In DT-40 3ko cells expressing wild-type $S2^-$ $InsP_3R$-1, incubation with 20 μM forskolin for 10 min resulted in a marked potentiation of the initial CCh-induced Ca^{2+} peak (fig 3A, pooled data fig 3E) in a similar fashion to that demonstrated for the $S2^+$ $InsP_3R$-1. In contrast to the $S2^+$ variant of the $InsP_3R$-1, neither single mutation in amino acids corresponding to S1589 or S1755 in $S2^+$ $InsP_3R$-1 resulted in loss of this enhanced Ca^{2+} signal (fig 3C/3D and 3E). These data indicated that both serine residues can be phosphorylated in this form of the receptor and furthermore was consistent with the observation that the peripheral $InsP_3R$-1 is more readily phosphorylated by PKA than the neuronal form [7]. However, no potentiation was observed in cells transfected with a double mutant where both serines were mutated to alanine (S1589A/S1755A $S2^-$ $InsP_3R$-1), confirming that no additional functionally important phosphorylation sites are present in the $S2^-$ $InsP_3R$-1 (fig 3D and 3E).

The degree of potentiation appeared similar when comparing wild-type to either S1589A or S1755A $S2^-$ $InsP_3R$-1; expression of each construct revealed a ~3 fold increase in CCh-induced Ca^{2+} release in the presence of forskolin. These data indicated, that if the assumption is made that phosphorylation of each particular site occurred independently, that phosphorylation of individual sites appeared not to result in an additive effect on Ca^{2+} release. A potential exists, however, that the dye used in these experiments (Fura-2; kd ~150 nM) is saturated with Ca^{2+} upon CCh exposure in the presence of forskolin, thereby masking any additive effect of phosphorylating both sites. For this reason, similar experiments were performed using the lower affinity Ca^{2+} indicator Fura-2-FF (kd ~ 10 μM). While the degree of potentiation was somewhat greater in cells expressing wild-type $S2^-$ $InsP_3R$-1 (~4.5 fold) there was no significant difference in the extent of enhancement when comparing wild-type to S1589A and S1755A expressing cells (Fig 3E; filled bars). These data indicate that while it is possible that Fura-2 measurements may indeed underestimate the degree of PKA-induced enhancement of Ca^{2+} release resulting from PKA phosphorylation, our data suggests that phosphorylation of individual sites appears not to be functionally additive.

To assess if the phosphorylation of a particular site was favored in $S2^-$ $InsP_3R$-1, the minimal concentration of forskolin sufficient to enhance CCh-induced Ca^{2+} release in wild-type vs. S1589A and S1755A mutants was compared. In cells transfected with wild-type $S2^-$ $InsP_3R$-1, 100 nM, 500 nM or 1 μM forskolin failed to enhance the subsequent CCh-induced Ca^{2+} release (3 experiments, >5 cells for each condition). Incubation with 5 μM forskolin resulted in a 1.26 ± 0.21 fold potentiation of Ca^{2+} release (n=6). At this threshold concentration of forskolin a similar degree of potentiation was observed in both S1589A (1.36 ± 25 fold; n=8) and S1755A (1.37 ± 0.26 fold; n=7) $S2^-$ $InsP_3R$-1. Thus, functionally, it appears that a particular site is not obviously subject to preferential phosphoregulation by PKA.

These data demonstrated that, in contrast to the $S2^+$ variant, both S1589 and S1755 are functionally important phosphorylation substrates in the $S2^-$ $InsP_3R$. This indicates that excision of the 40 amino acids in the $S2^-$ form of the $InsP_3R$-1 either alters the structure of the receptor allowing access to the kinase or perhaps allows the interaction with an accessory protein necessary to confer this effect. The only known structural difference between the splice variants is the insertion of an adenine nucleotide binding site in the $S2^-$ $InsP_3R$-1.

Phosphorylation of $S2^+$ $InsP_3R$-1 by PKG:

Similar experiments were performed to assess the effects on Ca^{2+} release of phosphorylating $InsP_3R$-1 with PKG. Cells transfected with $S2^+$ $InsP_3R$-1 together with M3 receptor and HcRed were stimulated with low, threshold concentrations of CCh (25-50 nM) followed by a 10 min treatment with 10 μM 8-Br cGMP, a specific activator of PKG [48]. Subsequent re-stimulation with an identical concentration of CCh revealed a marked potentiation of the $[Ca^{2+}]_i$ change. In a similar fashion to PKA activation, this was manifested as an increase in the CCh-induced initial peak (fig 4A and pooled data fig 4E). Moreover, these data are again consistent with phosphorylation increasing the sensitivity of the $InsP_3R$ to $InsP_3$, since sub-threshold increases in $[Ca^{2+}]_i$ were readily potentiated to substantial increases in $[Ca^{2+}]_i$ (fig 4A 4B and 4E).

Next, the effect of phosphorylation of S1589 and S1755 in $S2^+$ $InsP_3R$-1 mutants was assessed. In cells transfected with S1589A a similar potentiation by 8-Br cGMP was observed (fig 4B and 4E) while no enhanced $[Ca^{2+}]_i$ signal was observed in cells transfected with S1755A (fig 4C and 4E), strongly indicating that phosphorylation of S1755 by PKG is the important event underlying this potentiation of Ca^{2+} signaling. Although PKG and PKA

consensus motifs are similar (RRXS) these data are consistent with reports that S1755 is phosphorylated by PKG and furthermore that phosphorylation by PKG is enhanced by the presence of an aromatic amino acid, 4 amino acids downstream from the phosphorylatable serine as is the case for S1755 in $InsP_3R$-1 [49]. Indeed the substantial degree of potentiation may reflect the favorable nature of this site for phosphorylation by PKG. Although phosphorylation of S1755 appears to be responsible for the potentiated signal, our data thus far does not exclude the possibility that the response of wild-type $S2^+$ $InsP_3R$-1 is a result of a net phosphorylation of both serines by PKG, with phosphorylation of S1589 actually resulting in inhibited release. Thus, the effect of PKG activation on larger $[Ca^{2+}]_i$ responses to CCh stimulation was tested in cells transfected with S1589A $InsP_3R$-1. Using this paradigm no effect of 10 μM 8-Br cGMP was observed (fig 4D), confirming that either S1589 is not phosphorylated or has no functional consequence in $S2^+$ $InsP_3R$-1.

Although the most likely consequence of 8-Br cGMP treatment was to activate PKG, it was possible that this compound led to cAMP accumulation and activation of PKA indirectly by inhibiting cAMP phosphodiesterase [50]. Additionally, a somewhat less likely scenario was that 8-Br cGMP bound to and activated PKA directly. Experiments were therefore performed to confirm that the potentiation of Ca^{2+} signaling observed with 8-Br cGMP treatment was not the result of PKA activation. Cells transfected with $S2^+$ $InsP_3R$-1 were preincubated for 30 minutes with the cell-permeable PKA inhibitor, myristolyated PKI. This treatment completely abolished any potentiation of the CCh-induced $[Ca^{2+}]_i$ elevation after forskolin treatment (fig 5A and pooled data fig 5C). In contrast, similar treatment with PKI did not alter the potentiation induced by 8-Br cGMP treatment (fig 5B/C); this treatment still resulted in a ~20 fold potentiation of the CCh-stimulated $[Ca^{2+}]_i$ signal (compare fig 4A and fig 5B). These data clearly indicate that treatment with forskolin and 8-Br cGMP resulted in the selective activation of PKA or PKG respectively.

Phosphorylation of $S2^-$ $InsP_3R$-1 by PKG:

PKA or PKG phosphorylation of the $S2^-$ $InsP_3R$-1 in megakaryocytes and smooth muscle cells has been suggested to inhibit Ca^{2+} release [21, 36, 37]. This observation is difficult to reconcile with the data presented herein for phosphorylation of $S2^-$ $InsP_3R$-1 by PKA, since phosphorylation of either S1589 or S1755 resulted in potentiated release. Nevertheless, similar experiments were performed to elucidate the effect of PKG phosphorylation of $S2^-$ $InsP_3R$-1. Interestingly, no effect of 10 μM 8-Br cGMP treatment was observed in cells transfected with either wild-type (fig 6A and pooled data fig 6D),

S1589A S2⁻ $InsP_3R$-1 (fig 6B and 6D) or S1755A S2⁻ $InsP_3R$-1 (fig 6C and 6D). However, in the same batches of cells transfected with wild-type S2⁻ $InsP_3R$-1, treatment with forskolin resulted in the expected ~4 fold increase in the initial peak (n=5 cells). These data also reinforced the contention that 8-Br cGMP specifically activates PKG without altering PKA activity; the logic being that if, this was not the case, activation of PKA by 8-Br cGMP in S2⁻ $InsP_3R$-1 expressing cells would be expected to result in data similar to PKA activation shown in Fig 3.

The data demonstrated that PKA phosphorylation of either S1589 or S1755 resulted in enhanced Ca^{2+} release in the S2⁻ variant of the receptor (fig 3 A/B/C). Thus, PKG is simply not capable of *directly* phosphorylating this receptor. These data, although somewhat surprising, do not rule out the possibility that phosphorylation of an accessory protein termed IRAG [51, 52] may have an effect.

In conclusion, using mutational analysis in a *null* $InsP_3R$ background this study has elucidated the serine residues in $InsP_3R$-1 functionally important for modulating Ca^{2+} release by cyclic nucleotide dependent protein kinases. An important finding of this study was that, although phosphorylation of either S1589 or S1755 can result in markedly enhanced Ca^{2+} release, the particular splice variant of $InsP_3R$-1 expressed dictated which sites were susceptible to phosphorylation. Potentiation of Ca^{2+} release through $InsP_3R$-1 phosphorylation thus provided a powerful means of enhancing and amplifying Ca^{2+} signaling events when multiple signaling pathways are activated. Additionally, the evidence indicated that PKG appears not to directly regulate S2⁻ $InsP_3R$-1. Therefore, this splice variation defined which kinase is capable of phosphorylating the receptor at these sites and thus the specificity of functional response.

Example 2: Acute Regulation of Secretion: Cross-Talk Between Signaling Molecules and Their Effectors

Effects on $InsP_3$-induced Ca^{2+} release

Phosphorylation of inositol 1,4,5 trisphosphate receptors ($InsP_3R$) is a major point of synergism between the Ca^{2+} and cAMP signaling systems [29]. Phosphorylation of $InsP_3R$ in parotid acinar cells results in markedly enhanced Ca^{2+} release, an effect attributed to type-II $InsP_3R$ [29] . The functionally important phosphorylation sites have recently been defined in the type-I receptor [57]. The experiments examine the potential for "phosphomimetic" mutants of $InsP_3R$-1 to enhance Ca^{2+} signaling and the mechanism responsible for this augmentation of Ca^{2+} release in both the $InsP_3R$-I and $InsP_3R$-II. These

constructs can be utilized in models of impaired fluid secretion to assess their ability to augment fluid secretion.

Fluid secretion in salivary acinar cells

Appropriate control of salivary secretion is required for effective speech, mastication and general oral health [62-64]. Disruption of normal secretion is thus a significant health problem for affected individuals. The inability to produce an adequate salivary fluid secretion results in a variety of conditions that together comprise a major health problem for a significant proportion of the population [62, 63].

Fluid secretion in the salivary glands relies on the secondary active transepithelial transport of Cl^- [61, 65-68]. Briefly, Cl^- ions enter the cell across the basolateral membrane, in part via Cl^-/HCO_3^- exchange but primarily by an electrically neutral Na^+-K^+-$2Cl^-$ cotransport process [69-72]. The accumulated K^+ ions recycle to the serosal side via basolateral K^+ channels, whilst the accumulated Cl^- exit to the mucosal side via apical Cl^- channels. The resultant transepithelial movement of Cl^- generates an electrical potential gradient (lumen negative) sufficient to drive Na^+ into the lumen via a paracellular pathway. The net result is the secretion of Na^+ and Cl^-, with water following osmotically [61, 65, 68]. The principal means of regulation of this fluid secretion involves stimulation via parasympathetic nerves supplying the glands [73]. It is generally acknowledged that the main component of this system involves neurally released acetylcholine (ACh) acting at muscarinic receptors on the acinar cells [74-76]. Activation of these receptors produces a rise in $[Ca^{2+}]_i$ as a result of an increased turnover of membrane phosphoinositides and the generation of inositol 1,4,5-trisphosphate ($InsP_3$) [77]. The elevated $[Ca^{2+}]_i$ in turn acts at membrane ion channels, specifically increasing basolateral K^+ conductance and an apical Cl^- conductance [61, 65, 67, 68]. It is the increases in these conductance pathways that lead to the initiation of the secretion of ions and accompanying fluid. The rise in $[Ca^{2+}]_i$ also apparently has important additional effects including the stimulation of the activity of the basolateral Na^+-K^+-$2Cl^-$ cotransport increasing the entry of Cl^- ions into the cell [78].

Ca^{2+} signaling in parotid acinar cells

Many studies have investigated the characteristics of $[Ca^{2+}]_i$ signals in parotid acinar cells [79]. In common with other electrically non-excitable cells, cytosolic Ca^{2+} signals comprise both the release of Ca^{2+} from intracellular stores and the activation of pathways mediating the entry of Ca^{2+} from the extracellular space. It should be noted that while the release of intracellular Ca^{2+} is capable of initiating fluid secretion in the salivary glands, the ability to produce a sustained secretion is known to be entirely dependent on the influx of

Ca^{2+} from the extracellular medium [58-60]. Consequently, a thorough understanding of the processes leading to activation and modulation of both Ca^{2+} release and Ca^{2+} influx is fundamentally important for understanding fluid secretion.

The pathways involved in Ca^{2+} release are relatively well defined; the binding of secretagogues such as ACh, substance P, noradrenaline and ATP (acting at P2Y receptors) to plasma membrane receptors, couples to the heterotrimeric G protein Gαq/11, resulting in activation of PLCβ and the subsequent formation of $InsP_3$. All three isoforms of $InsP_3R$ are expressed to varying degrees in rodent parotid tissue, with ~80% comprising the type-II ($InsP_3R$-II) or type-III isoform ($InsP_3R$-III) and with $InsP_3R$-II constituting the absolute majority [80, 81]. Binding of $InsP_3$ to these receptors located in the endoplasmic reticulum results in explosive Ca^{2+} release into the cell cytoplasm. The vast majority of $InsP_3R$ are expressed in the extreme apical pole of the cells and this localization reasonably explains why Ca^{2+} release is initiated in this region [82, 53]. Physiologically, the initial increase in $[Ca^{2+}]_i$ is ideally situated to activate Ca^{2+}-dependent Cl^- channels which are localized exclusively to the luminal membrane [54]. Subsequently, the $[Ca^{2+}]_i$ signal rapidly becomes "global", facilitating the activation of basolateral Ca^{2+}-activated K^+ channels [80]. The process responsible for the extremely rapid globalization of the Ca^{2+} signal appears to be distinct from other exocrine cells, such as pancreatic acinar cells [80], in that the increase is much too rapid to be mediated by a classical Ca^{2+} wave propagated by sequentially Ca^{2+}-induced Ca^{2+} release from neighboring release sites . Instead, experimental evidence and mathematical modeling indicate that this phenomenon is best explained by largely autonomous, local Ca^{2+} release occurring throughout the cytoplasm mediated by both ryanodine receptors (RyR) and $InsP_3R$ [80, 55].

Importantly this secretagogue-induced Ca^{2+} release is modulated in both mouse and human parotid acinar cells under conditions where cAMP is elevated. cAMP results in a substantial increase in the $[Ca^{2+}]_i$ signal upon muscarinic stimulation relative to stimulation in the absence of cAMP. In particular the initial increase in $[Ca^{2+}]_i$ was enhanced, sub-threshold stimulation was transformed to a measurable $[Ca^{2+}]_i$ increase and an oscillatory increase was converted to a sustained $[Ca^{2+}]_i$ increase, all consistent with a left shift in sensitivity to stimulation by Ca^{2+} mobilizing agonists. Although, there are obviously many potential molecular targets of cAMP, these studies indicated that modulation by cAMP of PLC activity, pumping activity through PMCA or SERCA, and release through RyR did not appear to be responsible for the enhanced $[Ca^{2+}]_i$ signal [29, 57]. Instead the major effect

appeared to be enhanced Ca^{2+} release through PKA phosphorylation of $InsP_3R$. Moreover, this effect was attributed in particular to the $InsP_3R$-II since this isoform has the greatest sensitivity to $InsP_3$, is the most abundant in mouse parotid, and was shown to be specifically phosphorylated [29]. Since $InsP_3R$ are central to mobilizing $[Ca^{2+}]_i$ in parotid acinar cells, their regulation by phosphorylation provides a point of convergence whereby any secretagogues acting through Gq-PLC are subject to modulation. Indeed, this appears to be the case since Ca^{2+} release through P2Y purinoreceptors in mouse acini is also is enhanced by cAMP treatment.

While the functional consequences of $InsP_3R$ phosphorylation appear to be sub-type specific, recent studies in heterologous expression systems have demonstrated that phosphorylation of $InsP_3R$-I in addition to $InsP_3R$-II also leads to markedly enhanced Ca^{2+} release. The particular sites within $InsP_3R$-I which are functionally important for potentiated release have also been defined; two canonical PKA-phosphorylation motifs which are conserved from Drosophila to human are present in the $InsP_3R$-1 sequence but are not present in either the $InsP_3R$-II or $InsP_3R$-III [12]. In the splice variant of the $InsP_3R$-1 expressed in parotid acinar cells ($S2^-$ $InsP_3R$-1 or "short form") [7] phosphorylation of both sites occurs, resulting in $InsP_3R$ with an enhanced apparent sensitivity to $InsP_3$. Because potentiation of Ca^{2+} release at the level of $InsP_3R$ can be a nexus for interactions between the cAMP and Ca^{2+} signaling systems in parotid acinar cells, this project investigates the mechanism underpinning this effect by studying $InsP_3R$ with phosphomimetic mutations. These receptors can be used therapeutically to maximize $[Ca^{2+}]_i$ signals if expressed in diseased salivary tissue.

$InsP_3$-dependent Ca^{2+} release: *cAMP effects on Ca^{2+} release*

In mouse parotid acinar cells raising cAMP profoundly potentiates muscarinic agonist-induced $[Ca^{2+}]_i$ signals [29, 83, 84]. A major mechanism underlying this phenomenon can be the PKA-dependent phosphorylation of $InsP_3R$ [29]. The primary evidence supporting this is that cAMP elevation results in both $InsP_3R$ phosphorylation and a marked potentiation of Ca^{2+} release in a PKA dependent manner [29]. Data, consistent with the idea that $InsP_3R$ are central to this effect, is presented in Fig. 7. First, Ca^{2+} release generated through other Gq/PLC-linked receptors, such as P2Y purinergic receptors were also enhanced by cAMP elevation (Fig. 7A). Secondly, the effect was not species specific since a similar marked potentiation of the CCh-induced elevation in $[Ca^{2+}]_i$ was observed in human parotid acini incubated with either forskolin (Fig. 7B) or β-adrenergic agonist prior to stimulation. The effects of $InsP_3R$ phosphorylation can be subtype-specific. Enhanced

Ca^{2+} release is consistent with an effect on $InsP_3R$-II as this $InsP_3R$ is most abundant in parotid tissue and PKA-dependent phosphorylation of $InsP_3R$-II has been shown to augment Ca^{2+} release in other tissues such as liver [30]. It should be noted however, that all three subtypes of $InsP_3R$ are expressed in parotid tissue with similar localization to the apical domain of the cell.

Experiments expressing in the DT-40 3kocells a mutant $InsP_3$ R-1 where serine residues at position 1589 and 1755 are mutated to glutamate as described above, result in markedly potentiated Ca^{2+} release (Fig. 8).

Phosphorylation of $InsP_3R$ can alter apparent sensitivity to $InsP_3$ by a number of mechanisms. These include altering the sensitivity to $InsP_3$ itself, by changing the sensitivity of the receptor to its co-agonist Ca^{2+} or alternatively by modulating the interaction with other regulatory factors such as proteins or adenine nucleotides. Although studying Ca^{2+} release in DT-40 3ko cells as described above provides a powerful system to assess the net cellular effect of phosphorylation, a complimentary approach can be employed to study the biophysical properties of single $InsP_3R$ channels in isolated nuclei from cells overexpressing $InsP_3R$ and mutants. This technique relies on the fact that the outer nuclear membrane is continuous with the ER and can be patch clamped in the "on nucleus" configuration [11, 85-89]. Studies have successfully recorded $InsP_3$ dependent currents from isolated Cos-7 nuclei overexpressing $InsP_3R$-1 (Fig. 9). Because of the very low expression of endogenous $InsP_3R$ in Cos-7 cells [11, 88] $InsP_3$-dependent channel activity in untransfected/mock transfected cells has not been observed. In the example shown in Fig. 9, representative sweeps are shown from a single nucleus which was patched on 6 separate occasions; initially with saturating $InsP_3$, subsequently with no $InsP_3$, and then later again with $InsP_3$ in the patch pipette.

Example 3: Modulation of Ca^{2+} Release by Inositol 1,4,5-trisphosphate Receptor Phosphorylation

Inositol 1,4,5-trisphosphate receptors ($InsP_3R$) are the major route of intracellular calcium release in eukaryotic cells and thus are pivotal for stimulation of Ca^{2+} dependent effectors important for the control of numerous physiological processes (Figures 10 and 11). Modulation of Ca^{2+} release through $InsP_3R$ is thus of general importance for defining the particular spatio-temporal characteristics of Ca^{2+} signals. While it is widely appreciated that Ca^{2+} itself is an important regulator of $InsP_3R$, the receptor is also subject to modulation through numerous inputs, including protein-protein interactions, binding of adenine nucleotides and phosphorylation by multiple kinases. In this study, the effects on Ca^{2+}

release of phosphorylation of $InsP_3R$ by cyclic nucleotide-dependent protein kinases was studied.

To investigate the particular sites of phosphoregulation in an unambiguously homogenous population of homomeric receptors, type-1 $InsP_3R$ ($InsP_3R$-I) were expressed in DT-40 cells devoid of endogenous $InsP_3R$ (Figure 1A) (43). In cells expressing the neuronal, $S2^+$ splice variant of the $InsP_3R$-1, Ca^{2+} release was markedly enhanced when either PKA or PKG was activated (Figure 1B-D and as described above) (23). The sites of phosphorylation were investigated by mutation of serine residues present in two canonical phosphorylation sites present in the protein. Potentiated Ca^{2+} release was abolished when serine 1755 was mutated to alanine (S1755A) but was unaffected by a similar mutation of serine 1589 (S1589A) (Figures 3 and 12). These data demonstrate that S1755 is the functionally important residue for phosphoregulation by PKA and PKG in the neuronal variant of the $InsP_3R$-1. Activation of PKA also resulted in potentiated Ca^{2+} release in cells expressing the non-neuronal, $S2^-$ splice variant of the $InsP_3R$-1. However, the PKA-induced potentiation was still evident in S1589A or S1755A $InsP_3R$-1 mutants. The effect was abolished in the double (S1589A/S1755A) mutant, indicating both sites are phosphorylated and contribute to the functional effect (Figure 8). Indeed, mimicking phosphorylation by changing either S1589 or S1755 to a positively charged glutamate residue (S1589E or S1755E) resulted in $InsP_3R$ with apparent enhanced sensitivity to $InsP_3$ (Figures 8 and 13). Activation of PKG had no effect on Ca^{2+} release in cells expressing the $S2^-$ variant of $InsP_3R$-1. Collectively these data indicated that phosphoregulation of $InsP_3R$-1 had dramatic effects on Ca^{2+} release and defined the molecular sites phosphorylated in the major variants expressed in neuronal and peripheral tissues.

Example 4: Functional Consequences of Phosphomimetic Mutations at Key cAMP-dependent Protein Kinase Phosphorylation Sites in the Type I Inositol 1,4,5-Triphosphate Receptor

Two protein kinase A (PKA) consensus sites (RRXS) at Ser-1589 and Ser-1755 are present in the $InsP_3R$-1 (7, 99), and the most recent studies suggest that phosphorylation of these sites results in a marked enhancement of Ca^{2+} release (23, 24, 26, 100). Most interestingly, these sites are conserved through evolution from *Drosophila* to humans in $InsP_3R$-1, but corresponding sites are not present in either the InsP3R-2 or $InsP_3R$-3. It should be noted, however, that other regions, which are presently not defined, appear to function as PKA phosphorylation sites in $InsP_3R$-2 and $InsP_3R$-3. Several reports have demonstrated biochemically that both Ser-1589 and Ser-1755 can be phosphorylated in

$InsP_3R$-1 (34, 35, 99); however, these studies have not provided a consensus as to which sites are important physiologically in tissue that expresses either of the two major splice variants of $InsP_3R$-1.

These studies were performed by using mutational analysis by substituting alanine for the serine residues present at individual putative phosphorylation sites. Although phosphorylation of both neuronal (S2+) and peripheral (S2-) forms of $InsP_3R$-1 by PKA resulted in enhanced Ca^{2+} release, mutational analysis indicated that only phosphorylation of Ser-1755 was functionally important in the neuronal S2+ InsP3R-1. In contrast, both Ser-1589 and Ser-1755 appeared to be phosphorylated and significant in the peripheral S2- form of $InsP_3R$-1. In addition, although the S2+ form of the receptor was subject to direct phosphoregulation by cGMP-dependent protein kinase (PKG), the S2- form was not influenced by activation of PKG. These data represent one of the few major differences reported for the regulation of the two major splice variants of the $InsP_3R$-1.

Herein, charge mutations were constructed, substituting glutamate residues for the serine residues in the functionally important phosphorylation sites in both S2+ and S2- variants of the $InsP_3R$-1. These mutations can mimic phosphorylation and allow the assessment of the functional effects of phosphorylation of $InsP_3R$-1. Most importantly, the Ca^{2+} release properties of phosphomimetic mutations can be essentially independent of cell typespecific factors, including the expression of accessory proteins such as protein A-kinase anchoring proteins. These effects can be unambiguously specific to $InsP_3R$ and thus independent of confounding PKA effects on other Ca^{2+}-handling machinery. This latter consideration has historically plagued the functional assessment of PKA phosphorylation of $InsP_3R$. These mutations have allowed us to define the relative sensitivity of Ca^{2+} release of the phosphomimetic mutations and to confirm which sites are important in each splice variant.

The present study has also addressed whether phosphorylation of individual sites is permissive or additive in each splice variant, and the consequences of phosphorylating $InsP_3R$-1 on Ca^{2+} oscillations, the physiological pattern of Ca^{2+} signaling in nonelectrically excitable cells was investigated.

Materials and Methods

The acetoxymethyl esters of Fura-2 and Fluo-4 were purchased from Molecular Probes (Eugene, OR). Cell-permeable cyclic nucleotides and forskolin were purchased from Biomol (Plymouth Meeting, PA). All other chemicals were purchased from Sigma. The Dt-

40 cells lacking InsP3R (Dt-40 3ko) were kindly provided by Dr. Kurosaki (Kansai Medical University, Japan) and were maintained as described previously (42-44).

Production of Mutations

The rat S2+ $InsP_3R$-1 in the expression plasmid pIRES-GFP was digested with the restriction endonuclease SalI. The overhang created by digestion was blunted by using T4 polymerase. An EcoRI linker was then ligated onto the blunted ends of the construct. The entire receptor DNA was excised from the plasmid using EcoRI and ligated into the plasmid MXT-1. The region containing the S2- splice variant and potential PKA phosphorylation sites was excised from its backbone in pCDNA 3.1+ by RsrII and KasI and ligated into the InsP3R construct in MXT-1. The potential PKA phosphorylation sites Ser-1589 and Ser-1755 were mutated, individually in both splice variants and together in the S2- splice variant, to alanines or glutamates by using sequential PCR mutagenesis. The outside primers used for the mutagenesis reaction flanked the restriction sites RsrII and KasI. Following mutation, the resulting fragments were cut with RsrII and KasI and inserted into the $InsP_3R$-1 backbone at the corresponding sites. The mutations were confirmed by Big Dye fluorescent sequencing. Mutated receptor DNAs were excised from MXT-1 by using EcoRI and ligated into the mammalian expression vector pGW (provided by Dr. David Yue, Johns Hopkins University). Orientation was confirmed by using restriction enzyme digestion. Mutants were named based on the splice variant, either S2+ or S2- followed by the amino acid present at position 1589 and 1755. Thus, a mutation in S2+ $InsP_3R$-1 S1755E is designated,"S2+ SE," and in $InsP_3R$-1 S2+ S1755A is designated "S2- SA". Similarly, an S2- $InsP_3R$-1 with mutations in both S1589E and S1755E is designated "S2-EE". The numbering of residues is based on the full-length rat $InsP_3R$-1.

Transfection of Dt-40 Cells

Dt-40 cells lacking all three $InsP_3$ receptor subtypes were transfected by using electroporation at 350 V and 950 microfarads (4-mm gap cuvette). $2x10^7$ cells were co-transfected with 25 μg of the InsP3R-1 cDNA, 25 μg of the muscarinic type 3 (m3R) receptor DNA, and 4 μg of the red fluorescent protein plasmid pHcRed1-N1 (Clontech). Cells were incubated with DNA in 500 μl of Opti-MEM media (Invitrogen) on ice for 10 min. The cell/DNA mixture was electroporated, incubated on ice for 30 min, increased to 5 ml with Opti-MEM, and placed in a 5% CO_2 incubator at 39 °C for 5 h. The cells were then centrifuged and resuspended in 12 ml of complete RPMI media (Invitrogen). Transfection efficiency was typically ~20%. Experiments were performed within 32 h of transfection.

Transfection of HEK-293 Cells and Assessment of Phosphorylation of S2- $InsP_3R$-1

HEK-293 cells were plated onto 25-cm^2 culture flasks and allowed to grow to near-confluency. Cells were transfected with 5 μg of each S2- $InsP_3R$-1 DNA construct by using the LipofectAMINE reagent (Invitrogen) as per the manufacturer's instructions. The following day, batches of cells were treated in the presence or absence of 20 μM forskolin for 10 min, aspirated from flasks, lysed, and immunoprecipitated with a polyclonal α-InsP3R-1 antibody that recognizes amino acids 2731–2749 of $InsP_3R$-1. Immunoprecipitates were separated on 5% SDS gels transferred to nitrocellulose and then probed with either the α-$InsP_3R$-1 antibody or a polyclonal antibody that recognizes the phosphorylated state of Ser-1755 (39) (α-phospho-Ser-1755), kindly provided by Dr. S. Snyder. Blots that were probed with α-phospho-Ser-1755 were stripped and reprobed with the α-$InsP_3R$-1 antisera to confirm the presence and relative quantity of the $InsP_3R$-1.

Digital Imaging of $[Ca^{2+}]_i$

Transfected Dt-40 3ko cells were washed once in a HEPES-buffered physiological saline solution (HEPES-PSS) containing (in mM) 5.5 glucose, 137 NaCl, 0.56 MgCl2, 4.7 KCl, 1 Na2HPO4, 10 HEPES (pH 7.4), 1.2 CaCl2, and 1% w/v bovine serum albumin. Cells were then resuspended in bovine serum albumin HEPES-PSS with 1 μM Fura-2 (AM), placed on a 15-mm glass coverslip in a low volume perfusion chamber, and allowed to adhere for 30 min at room temperature. Cells were perfused continuously for 10 min with HEPES-PSS before experimentation to allow complete Fura-2 de-esterification. A field of cells for each experiment was chosen that contained a wide range of transfection efficiency based upon the intensity of red fluorescence emitted when excited at 560 nm. Individual cells that had emission gray levels between 1500 and 2500 were subsequently chosen to standardize expression levels. $[Ca^{2+}]_i$ imaging was performed essentially as described previously by using an inverted epifluorescence Nikon microscope with a 40X oil immersion objective lens (numerical aperture, 1.3) (100). Cells were excited alternately with light at 340 and 380 ± 10 nm bandpass filters (Chroma, Rockingham, VT) using a monochrometer (TILL Photonics, Pleasanton, CA). Fluorescence images were captured and digitized with a digital camera driven by TILL Photonics software. Images were captured every 2 s with an exposure of 2 ms and 4 by 4 binning. 340/380 ratio images were calculated online and stored immediately to a hard disk.

"Flash" Photolysis

Transfected cells were simultaneously loaded with the visible wavelength indicator Fluo-4 and a cell-permeable form of caged inositol trisphosphate (ci-IP_3/PM) for 30 min. ci-IP_3/PM is a homologue of cm-IP_3/PM. The 2- and 3-hydroxyls of the inositol ring are protected by an isopropylidene group in ci-IP_3/PM and are protected by a methoxymethylene group in cm-IP_3/PM (45). Like cm-IP_3/PM, ci- IP_3/PM diffuses across cell membranes, and the PM group is hydrolyzed by cellular esterases, and Ca^{2+} release can be induced upon photouncaging as i-IP_3 is liberated from the cage and acts in a similar fashion to $InsP_3$ at $InsP_3R^2$. A further period of ~30 min was allowed for de-esterification of both dye and cage. Cells were illuminated at 488 ±10 nm and fluorescence collected through a 525 ± 25-nm bandpass filter and captured using the Till Photonics imaging suite. These traces are displayed as % $\Delta F/F_o$, where F is the recorded fluorescence, and F_o is the mean of the initial 10 sequential frames. Photolytic release was performed as described previously by using a pulsed xenon arc lamp (Till Photonics). A high intensity (0.5-5 ms duration; 80 J) discharge of UV light (360 ± 7.5 nm) was reflected onto the plane of focus by using a DM400 dichroic mirror and Nikon 40X oil immersion objective, 1.3 NA.

Frequency Distribution of Ca^{2+} Oscillations

Cells transfected with WT and mutant S2- isoforms were stimulated with varying concentrations of an α-IgM antibody (Southern Biotechnology Associates, Inc., Birmingham, AL). Infrequent Ca^{2+} oscillations were produced presumably through B cell receptor cross-linking, activation of phospholipase C-γ, and subsequent production of $InsP_3$. The frequency of Ca^{2+} oscillations was determined by selecting individual peaks that displayed an increase in ratio units greater than 0.05 and are listed as frequency in milliHertz (number of oscillations in 1000 s).

Concentration-Response Relationships

Normalized ΔF concentration- response relationships were fit with the following logistic.

Equation 1,

$$\Delta F = 1/(1 + (C/EC_{50})^{Slope\Delta F} \qquad \text{(Eq. 1)}$$

where ΔF is the change in fluorescence normalized to the maximal response; C is agent concentration; EC_{50} is the concentration where the response is half of maximum, and $Slope\Delta F$ is a slope factor related to the Hill coefficient.

Statistical Analysis

The effects of treatment were determined by normalizing the peak change in fluorescence ratio by stimulation following forskolin or 8-Br-cGMP exposure to that of stimulation in control HEPES-PSS. Thus, pooled data represent a normalized fold increase over control for the treated trial. In all cases where statistical significance is indicated, two-tailed heteroscedastic *t* tests were performed. *P* values < 0.05 were considered to indicate statistical significance and are denoted by an asterisk in the figures.

Phosphomimetic Mutations in Functionally Important Phosphorylation Sites in Both S2+ and S2- $InsP_3R$-1 Result in Enhanced Ca^{2+} Release

Phosphorylation of proteins results in the addition of net negative charge to the phosphoacceptor residue. In the case of PKA phosphoregulation, the functional effects of phosphorylation are thought to occur as the negative charge added to the serine or (less frequently) threonine residues neutralizes the positive charge of basic arginine or lysine residues present upstream in the classical RR*X*(S/T) consensus motif (101). This charge neutralization, in turn, is thought to result in a conformational change in the protein. A common approach employed to investigate the functional effects of phosphorylation is to construct phosphomimetic mutations whereby glutamic or aspartic acid residues are substituted at the phosphoacceptor site (102, 103). The rationale for this strategy is that the negatively charged side chain of the substituted acidic amino acid will mimic, to an extent, the addition of a phosphate moiety to the protein.

To investigate the consequences of $InsP_3R$-1 phosphorylation, the Ca^{2+} release properties of phosphomimetic mutations in the functionally important sites in both S2+ and S2- $InsP_3R$-1 was analyzed in Dt-40 3ko cells. This cell line provides the only known InsP3R *null* background (42, 43). In initial experiments, a comparison was made between the sensitivity of Ca^{2+} release by $InsP_3R$-1 phosphomimetic mutations *versus* nonphosphorylatable alanine mutations at the sites. Dt-40 3ko cells were transfected with DNA encoding HcRed to facilitate identification of transfected cells and either S2-S1589E/S1755E $InsP_3R$-1 (S2- EE) or S2- S1589A/S1755A (S2- AA). Ca^{2+} release was monitored following flash photolysis of ci-IP_3, a cell-permeable form of caged $InsP_3$. This experimental paradigm provides a relatively direct assessment of the effects of $InsP_3R$-1 phosphorylation on the Ca^{2+} release process. The amount of ci-IP_3 photo-released was controlled by varying the duration of the UV flash discharge (0.5–5 ms). No increase in $[Ca^{2+}]_i$ was observed following the longest UV discharge in cells either not loaded with ci-IP_3/PM or not expressing HcRed. Fig. 17, *B* and *C*, shows traces from typical experiments

in individual Dt-40 3ko cells expressing S2- EE or S2- AA (Fig. 17, *B* and *C,* respectively, and pooled data in Fig. 17*F*). In cells expressing S2- EE, Ca^{2+} release as defined by a >0.05% $\Delta F/F_o$ increase in initial fluo-4 fluorescence was observed in ~60% of cells when exposed to UV discharge for 0.5 ms. Subsequent exposure to UV light for 1.25 ms elicited a more robust increase in $[Ca^{2+}]_i$ in all cells. Finally, photo-release following a 5-ms flash, in general evoked a further increase in the magnitude of Ca^{2+} release. In contrast, an elevation of intracellular Ca^{2+} was never observed under identical conditions following a 0.5-ms UV flash in cells expressing S2- AA. A significant Ca^{2+} release was only observed in ~50% of cells following a 1.25-ms flash, and the majority of cells only responded to the longest uncaging duration, albeit with a smaller magnitude than in S2- EE-expressing cells exposed to the same stimulus.

A similar pattern of sensitivity was observed in Dt-40 3ko cells expressing S2+ S1755E (S2+ SE) and S2+ S1755A (S2+ SA) as shown in Fig. 17, *D* and *E,* respectively, and pooled data in Fig. 17*G*. Cells expressing S2+ SE responded more robustly to photolysis of ci-IP3 than cells expressing S2+ SA exposed to an identical stimulus. These data indicate that serine to glutamate mutations at the functionally important phosphorylation sites in both splice variants of $InsP_3R$-1 are "phosphomimetic," *i.e.* charge mutations mimic phosphorylation in that these constructs display an apparent increased functional sensitivity to $InsP_3$.

Sensitivity of S2- $InsP_3R$-1 and Phosphorylation Site Mutants

Experiments were next performed to determine the relative sensitivity of Ca^{2+} release through the phosphomimetic mutations of the S2- $InsP_3R$-1 with respect to the wild type and phosphorylation-deficient mutants. Dt-40 3ko cells were transfected with cDNAs encoding m3R, HcRed together with the $InsP_3R$-1 construct of interest. Stimulation with the muscarinic agonist CCh results in robust increases in $[Ca^{2+}]_i$ in transfected cells through the Gαq/11-coupled stimulation of phospholipase C-β and subsequent formation of InsP3. The magnitude of the initial peak provides a good estimation of the extent of Ca^{2+} release as this parameter in Dt-40 cells, like many cells, is essentially independent of Ca^{2+} influx. Furthermore, in Dt-40 cells the $[Ca^{2+}]_i$ response to stimulation with CCh does not appreciably desensitize, and thus the effects of multiple concentrations of agonist can be assessed in a single cell. Individual HcRed-expressing cells were stimulated with increasing concentrations of CCh (1 nM to1 μM) for 60 s followed by a 5-min wash between applications of agonist. In each case, these experiments were performed on multiple cells,

expressing a narrow range of HcRed fluorescence and from multiple batches of transfected cells to minimize variation because of expression level of m3R and $InsP_3R$-1. Concentration-response relationships were generated by normalizing each initial peak to the maximum response in the individual cell and subsequently averaging the pool of cells expressing a particular construct. Fig. 18*A* shows a typical example of this experimental procedure performed on Dt-40 3ko cells expressing S2- EE. A significant increase in Ca^{2+} was detected in the majority of S2- EE-expressing cells following stimulation with 1 nM CCh, and the peak response occurred following stimulation between 10 and 50 nM CCh. In contrast, as shown in Fig. 18, *B* and *C*, Ca^{2+} release following CCh stimulation in the wild type S2- $InsP_3R$-1 or S2- AA was considerably less sensitive to CCh stimulation. Analysis of the pooled data indicated that the magnitude of the peak response to any of these constructs was not significantly different (maximum peak Δ response: S2- WT =0.9±0.1 ratio units; S2- AA =0.67±0.1 ratio units, and S2- EE =1.13±0.1 ratio units), indicating that the efficacy of Ca^{2+} release was essentially unaltered. However, when an estimate of the relative sensitivity was made by fitting the normalized concentration-response relationships for each construct (Fig. 18*D*), CCh-induced Ca^{2+} release in the S2- EE mutant (EC_{50} 4.3±1.2 nM CCh) was 7.5-fold more sensitive when compared with WT S2- $InsP_3R$-1 (EC_{50} =32.6±7.5 nM) and some 50-fold more sensitive than the nonphosphorylatable S2- AA mutant (EC_{50} =229.5±14.6 nM). These data provide strong evidence that the S2- EE construct is more sensitive to stimulation by $InsP_3$ and present evidence that phosphorylation of the $InsP_3R$-1 results in marked regulation of channel function. This profound regulation could be expected to have major consequences for calcium signaling events in peripheral tissue such as liver, testis, and smooth muscle which express the S2- $InsP_3R$-1 (7, 90). The observation that S2- AA is relatively less sensitive than S2- WT is consistent with the possibility that a proportion of the wild type receptor is constitutively phosphorylated in Dt-40 cells, thus contributing to the intermediate sensitivity of the wild type S2- $InsP_3R$-1 relative to the phosphomimetic S2- $InsP_3R$-1 receptor.

Although it has been demonstrated that each site can be phosphorylated, it is not definitively known whether the functional effects of phosphorylating individual sites are independent and additive or alternatively if the full effect is seen following phosphorylation of an individual site. Thus, experiments were next performed to assess the sensitivity of single phosphomimetic mutations within each phosphorylation site. Concentration-response relationships for CCh-induced Ca^{2+} release were constructed for Dt-40 3ko cells expressing either S2- S1755E $InsP_3R$-1 (S2- SE) or S2- S1589E $InsP_3R$-1 (S2- ES). The

PCT/US05/02380
data were analyzed as described previously for Fig. 18*D*. Fig. 19*A* shows the fit for the normalized concentration-response relationship for S2- SE and S2- ES and for comparison also shows the fit for S2- EE and S2- WT (Fig. 19*A, dotted lines*; data from Fig. 18*D*). Once again the maximal initial peak responses in either mutant were not significantly altered from wild type (S2- ES =0.54±0.1 Δ ratio units; S2- SE =0.78±0.1 Δ ratio units); however, the sensitivity of each of these mutants was significantly shifted, such that the EC_{50} for CCh-induced Ca^{2+} release was enhanced ~3-fold over the response in wild type for either mutant (EC_{50} for CCh-induced release: S2-ES =12.4±0.5 nM; S2- SE =13.5±1 nM). These data are summarized in Table 2.

The enhanced apparent sensitivity of individual phosphomimetic mutants was essentially equal and intermediate between the sensitivity of the S2- WT and S2- EE mutations. These data are consistent with phosphorylation of individual sites being functionally additive. To address this possibility directly, experiments were performed evaluating the effects of activating endogenous PKA in cells expressing S2- SE or S2- ES to mimic prior phosphorylation of an individual site. As shown in Fig. 19*B*, and reported previously, activation of PKA by incubation with forskolin results in a dramatic potentiation of Ca^{2+} release in Dt-40 3ko cells expressing S2- WT (100). Most surprisingly, although the sensitivity to CCh was enhanced, as evidenced by the low concentration of CCh necessary to evoke threshold Ca^{2+} release, no potentiation of Ca^{2+} release following forskolin incubation was observed in Dt-40 3ko cells expressing either S2- ES (Fig. 19*C*; pooled data in Fig. 19*F*), S2- SE (Fig. 19*D*; pooled data in Fig. 19*F*), or S2- EE (Fig. 19*E*; pooled data in Fig. 19*F*). Thus, although each phosphorylation site in S2- $InsP_3R$-1 can be phosphorylated and mimicking phosphorylation of both sites leads to a receptor with enhanced sensitivity relative to phosphorylation of an individual site, phosphorylation of both sites *in situ* does not appear to occur and therefore is not functionally additive. Although these data may seem paradoxical, an explanation, consistent with all the observations is that the initial phosphorylation of either Ser-1589 or Ser-1755 leads to a conformational change in the receptor that now precludes the phosphorylation of the additional site. To test this, experiments were performed to determine the phosphorylation state of the various mutants after raising cAMP levels. These experiments were performed in HEK-293 cells because of the high transfection efficiency and low endogenous levels of $InsP_3R$-1 (47, 99). HEK-293 cells were transfected with the constructs as indicated in Fig. 20, incubated in the presence or absence of forskolin for 10 min, then pelletted, and lysed. Following incubation of the lysates with α-$InsP_3R$-1 antibody, the immune complexes were

captured and separated on SDS gels, and the phosphorylation status of Ser-1755 was determined by Western blotting with an antibody that specifically recognizes this phosphorylated residue in InsP3R-1 (39). As shown in Fig. 20*A*, wild type S2- InsP3R-1 was robustly phosphorylated after forskolin incubation, whereas no phosphorylation was detected in wild type S2- AA or untransfected HEK-293 cells. Similarly, in Fig. 20*B*, a marked increase in phosphorylation could be detected in S2- $InsP_3R$-1, whereas no phosphorylation could be detected in cells transfected with S2- EE, S2- SE or, most importantly, the S2- ES construct. These data indicate that the initial phosphorylation of one site precludes further phosphorylation at the additional residue as indicated by the functional data and indicate that physiologically only phosphorylation of a single residue is functionally relevant in the S2- splice variant of $InsP_3R$-1.

Sensitivity of S2+ InsP3R-1 and Phosphorylation Site Mutants

The sensitivity of Ca^{2+} release via the neuronal S2+ $InsP_3R$-1 was next assessed by using a similar experimental paradigm to that used for the S2- form of the receptor. Although biochemically both Ser-1589 and Ser-1755 are equally susceptible to phosphorylation by PKA in S2+ $InsP_3R$-1 (99), only Ser-1755 appears to be functionally relevant in terms of modulating Ca^{2+} release (100). Fig. 21*A* shows the fits for the normalized concentration-response relationships for CCh-induced Ca^{2+} release in cells expressing S2+ WT, phosphomimetic S2+ SE, and S2+ SA. The apparent sensitivity of CChinduced Ca^{2+} release in cells expressing S2+ WT was essentially identical to S2- WT InsP3R-1 (EC_{50} for CCh-induced Ca^{2+} release; S2+ $InsP_3R$-1 =32.1±4.2 nM *versus* S2- 32.6±7.5 nM). These data indicate that $InsP_3$ binding and $InsP_3$-induced calcium release is identical in the two major splice variants of the $InsP_3R$-1 (104–106) and is therefore supportive of the contention that the initial CCh-stimulated $[Ca^{2+}]_i$ peak is a good indicator of $InsP_3R$ function. In a similar fashion to the S2- InsP3R-1, mutation of Ser-1755 to either alanine or glutamic acid did not significantly alter the maximal initial peak upon CCh stimulation (maximum peak Δ response: S2+ WT =0.61±0.1 Δ ratio units; S2+ SE =0.59±0.1 Δ ratio units; S2+ SA =0.84±0.2 Δ ratio units) but did, however, significantly affect the apparent sensitivity of Ca^{2+} release as shown in Fig. 21*A*. The S2+ SE mutant exhibited a similar EC_{50} for CChinduced Ca^{2+} release as the S2- EE mutation (EC_{50}: S2+ SE =3.6±0.2 nM CCh; S2- EE =4.3 nM), being ~9-fold more sensitive than S2+ WT and ~32-fold more sensitive than the nonphosphorylatable S2+ SA mutation (EC_{50} S2+ SA =116.3±3 nM CCh). These data are summarized in Table 2.

Table 2. *Summary of the effects of phosphorylation by PKA or PKG of the S2-/S2+ splice variants of $InsP_3R$-1 and phosphoregulatory mutations at Ser-1589 and Ser-1755 expressed in Dt40 3 knock out cells*
The data are derived from this paper and Ref. 100. NT, not tested.

Amino acid at 1589/1755	S2+ $InsP_3R$-1		S2- $InsP_3R$-1	
	CCh EC_{50} (M)/(fold change from WT)	Ca^{2+} release after PKA/PKG activation	CCh EC_{50} (M)/(fold change from WT)	Ca^{2+} release after PKA/PKG activation
Wild-type SS	32.1±4.2 / (x1)	Enhanced/enhanced	32.6±7.5/(x1)	Enhanced/no change
AS	NT	Enhanced/enhanced	NT	Enhanced/no change
SA	116.3±3.0 / (x0.3)	No change/no change	NT	Enhanced/no change
AA	NT	NT/NT	229.5±14.6/(x0.14)	No change/no change
SE	3.6±0.2 / (x8.9)	No change/no change	13.5±1.1/(x2.4)	No change/NT
ES	23.7±1.2 / (x1.3)	Enhanced/enhanced	12.4±0.5/(x2.6)	No change/NT
EE	NT	NT/NT	4.3±1.2/(x7.6)	No change/NT

PCT/US2005/002380

In contrast to the S2+ $InsP_3R$-1 S1755E construct, mutation of Ser-1589 to glutamate in the S2+ form of $InsP_3R$-1 did not significantly affect the apparent sensitivity of Ca^{2+} release as shown in Fig. 21*B*. Both the maximum peak response to CCh and the sensitivity of the receptor were very similar to that of S2+ WT $InsP_3R$-1 (maximum peak Δ response =0.59±0.2 Δ ratio units; EC_{50} =23.7±1.2 nM CCh). However, it is formally possible that phosphorylation of Ser-1589 is only functionally important following phosphorylation of Ser-1755 in S2+ $InsP_3R$-1. To test this, experiments were performed activating PKA with forskolin in Dt-40 3ko cells expressing S2+ ES or SE mutants. Treatment with forskolin resulted in a marked potentiation of CCh-induced Ca^{2+} release in cells expressing either S2+ WT or S2+ ES (Fig. 22, *A* and *B,* respectively, and pooled data in *D*) presumably as Ser-1755 was phosphorylated. Although cells expressing S2+ SE were more sensitive to CCh, no further potentiation was observed following forskolin incubation (Fig. 22*C* and pooled data in Fig. 22*D*). These data indicate that phosphorylation of Ser-1755 is permissive for any functional effect of phosphorylating Ser-1589 in S2+ $InsP_3R$-1.

Effect of PKG Activation on S2+ $InsP_3R$-1 and Phosphomimetic Mutants

Despite these differences, experiments were performed to define further the phosphorylation sites important for regulation of Ca^{2+} release by PKG. Fig. 23*A* shows a representative trace from cells expressing S2+ WT $InsP_3R$-1 thus illustrating the marked potentiation of Ca^{2+} release upon specific activation of PKG by 8-BrcGMP. A similar striking potentiation of Ca^{2+} release was also observed in cells expressing S2+ ES (Fig. 23*B* and pooled data Fig. 23*D*), again presumably as Ser-1755 is phosphorylated following incubation with 8-Br-cGMP. In contrast, in cells expressing S2+ SE no effect on CCh-induced Ca^{2+} release following activation of PKG was observed (Fig. 23*C* and pooled data in Fig. 23*D*). These data provide evidence that phosphorylation of Ser-1589 by PKG either in isolation or following phosphorylation of Ser-1755 plays a role in modulation Ca^{2+} release through S2+ $InsP_3R$-1.

The specific pattern of phosphorylation occurring upon stimulation of PKA or PKG can be cell type-specific as, for example, in the case of PKA as a result of the targeting through protein A-kinase anchoring proteins, as has been demonstrated recently (107) for $InsP_3R$. Alternatively, cell-specific effects could conceivably occur through restricted access of the kinase to its substrate. Notwithstanding the general importance of kinase targeting for efficient, localized phosphorylation, the phosphomimetic constructs used in this study reveal the intrinsic, functionally important sites in a manner independent of the particular cellular context because any targeting step is circumvented. These sites and the functional

PCT/US2005/002380
consequences of phosphoregulation can be a general property of the $InsP_3R$-1. In addition, the particular sites in S2+ $InsP_3R$-1 are entirely consistent with the earlier study (100) of the sites functionally important in enhanced Ca^{2+} release following PKA or PKG phosphoregulation, and this reinforces the view that phosphorylation of S2+ InsP3R-1 Ser-1589 has no significant role, at least in terms of Ca^{2+} release.

Effect of InsP3R Phosphorylation on Ca^{2+} Signaling Events

In many nonelectrically excitable cell types, the physiological mode of Ca^{2+} signaling is through the generation of Ca^{2+} oscillations (1, 108). Moreover, it is a generally held view that the spatial and temporal properties of Ca^{2+} oscillations make an important contribution to defining the fidelity and specificity of Ca^{2+} signaling. In many current models addressing the mechanism underlying Ca^{2+} oscillations, a key feature is the regulation of Ca^{2+} release through $InsP_3R$. Experiments were then designed to assess the consequences of $InsP_3R$-1 phosphorylation (expressed in isolation) on the initiation and generation of Ca^{2+} oscillations. An α-IgM antibody was chosen to stimulate and to initiate Ca^{2+} oscillations through activation of the endogenous B cell receptor, phospholipase C-γ activation, and the formation of $InsP_3$. Dt-40 3ko cells expressing either S2- WT or S2- EE to mimic PKA phosphorylation of the $InsP_3R$-1 were stimulated with various concentrations of α-IgM antibody as illustrated by the selection of representative traces in Fig. 24. Stimulation with 250 ng/ml α-IgM proved to be a threshold concentration in S2- WT-expressing cells. This degree of stimulation generally resulted in a single small increase in $[Ca^{2+}]_i$ after a long latency (Fig. 24*A, left panel,* and pooled data in Fig. 24, *C-E*). An identical stimulus in cells expressing S2- EE, in contrast, resulted in repetitive Ca^{2+} transients following a much shorter latency, consistent with the increased apparent sensitivity of the S2- EE constructs (Fig. 24*A, right panel,* and pooled data Fig. 24, *C-E*). Most interestingly, stimulation of S2- WT-expressing cells with 500 ng/ml α-IgM antibody resulted in Ca^{2+} oscillations with similar frequency to cells expressing S2- EE (Fig. 24*B* and pooled data *C-E*). However, the latency before the initiation of an increase in Ca^{2+} was significantly shorter in S2- EE-expressing cells. In addition, the magnitude of the initial transient was also significantly larger in S2- EE-expressing cells when compared with WT. Stimulation with 1 μg/ml α-IgM antibody resulted in transients that were indistinguishable in terms of latency, frequency, or initial peak magnitude in S2- EE- or S2- WT expressing cells (pooled data Fig. 24, *C-E*). Thus, PKA-mediated phosphorylation, by increasing the sensitivity of the $InsP_3R$-1 to $InsP_3$, can define the threshold at which cells begin to oscillate

but does not markedly influence the temporal properties of Ca^{2+} oscillations when initiated. This latter observation presumably reflects the fact that the frequency of oscillations is primarily defined by mechanisms such as Ca^{2+} feedback (109) rather than the absolute sensitivity of the $InsP_3R$-1 to $InsP_3$ within a defined range. These data are largely consistent with data from hepatocytes where PKA activation resulted in cells exhibiting a lower threshold for activation by $InsP_3$ infusion (110) or agonist activation (111). These data using phosphomimetic mutations of InsP3R-1 splice variants are in broad agreement with a number of studies that have reported increased sensitivity of $InsP_3R$-1 activity following phosphoregulation by PKA (23, 24, 26, 100, 111, 112). An important consequence of this increased sensitivity is defining the threshold where a cell will respond to a stimulus. Given the almost ubiquitous expression of various forms of $InsP_3R$-1, this is likely a generally important phenomenon. A number of mechanisms are plausible to explain the increased sensitivity of $InsP_3$-induced Ca^{2+} release. These include modulation of $InsP_3$ binding, an idea supported by measurements of $InsP_3R$ binding in hepatocytes (112) (presumably S2- $InsP_3R$-1 and $InsP_3R$-2). In these studies the apparent affinity of $InsP_3$ binding was enhanced ~2-fold at resting $[Ca^{2+}]$, and the $[Ca^{2+}]$ necessary for half-maximal stimulation of $InsP_3$ binding was reduced. In contrast, a study of recombinant S1- S2+ $InsP_3R$-1 expressed in SF9 cells and reconstituted into lipid bilayers has reported a similar increase in $InsP_3R$ sensitivity to $InsP_3$ but that the bell-shaped Ca^{2+} sensitivity of channel opening is not altered following phosphorylation of the $InsP_3R$-1 (23). These findings indicate that modulation of the Ca^{2+} sensitivity of channel activity is unlikely to account for the increased apparent sensitivity of the receptor, at least in this form of the $InsP_3R$-1. There are a number of alternative mechanisms for altering $InsP_3R$-1 sensitivity are conceivable. For example, phosphorylation of the receptor could alter the gating of the channel directly. In addition, phosphorylation might secondarily modulate the receptor by regulation of the association of regulatory factors such as proteins or adenine nucleotides (113–115). Indeed, a precedent for this type of regulation exists because PKA phosphorylation has been shown to alter the association of calmodulin with the S2- form of $InsP_3R$-1 (106).

Example 5: $InsP_3$ receptor function

While both $InsP_3R$-1 and $InsP_3$-2 phosphorylation can result in enhanced Ca^{2+} release, studies have focused on the $InsP_3R$-1, in particular in defining which sites are functionally important in the splice variant of the $InsP_3R$-1 ($S2^-$ variant) expressed in parotid acinar cells. Dt-403ko cells were transfected with $S2^-$ $InsP_3R$-1 and muscarinic m3 receptors (m3R), along with red fluorescent protein (Hc-Red) to identify transfected cells. After

loading with fura-2, treatment with forskolin was shown to result in a potentiated Ca^{2+} release in response to low concentrations of CCh (Fig. 25A) in a similar fashion to that observed in parotid acinar cells. Cells not transfected with either m3R or $InsP_3R$ cDNA failed to respond. Similarly, cells not expressing Hc-Red seldom responded to CCh treatment.

Since Dt-403ko cells are a genetically tractable system, this has allowed a mutagenic analysis of the specific sites important for PKA phosphoregulation in the $S2^-$ $InsP_3R$-1. Mutation of either potential phosphorylation site at serine 1589 or serine 1755 failed to attenuate this potentiation (Fig. 25B and 25C respectively). In contrast, mutation of both serine residues to alanine ("double alanine" or S2-AA mutation) completely abolished potentiation of the CCh-induced Ca^{2+} release by forskolin (Fig. 25D). These data indicate that both consensus motifs in the $S2^-$ $InsP_3R$-I are subject to PKA-dependent phosphoregulation, an observation which is in contrast to the neuronal isoform of the $InsP_3R$-I where only S1755 is subject to phosphoregulation. These experiments indicate that S1589 and S1755 constitute the only functional important PKA phosphorylation sites in $S2^-$ $InsP_3R$-2.

Because of its relative abundance in salivary tissue, and its high sensitivity to $InsP_3$, the $InsP_3R$-2 can be of key importance to salivary gland physiology. However, in the $InsP_3$ receptor field in general, only very limited progress regarding the unique structure and function of the $InsP_3R$-2 has been made. This is largely due to the difficulty of expressing this receptor subtype in a heterologous system. This problem was overcome by deletion of the 3' and 5' untranslated regions of the cDNA, and by inserting a kozak consensus sequence at the initiation site. As shown in Fig. 26, these manipulations have allowed for the successful expression of the optimized rat $InsP_3R$-2 in DT-403ko cells and, for the first time, to assess the effects of phosphorylation on an unambiguous homogeneous population of $InsP_3R$-2. Using a similar paradigm to that described for $InsP_3R$-1 (see Fig. 25) forskolin treatment results in a dramatic potentiation of carbachol-induced $[Ca^{2+}]_i$ signaling. Interestingly activation of the cGMP dependent protein kinase with 8Br-cGMP is without effect. Mutation of the consensus phosphorylation sites in the receptor can allow for the development of similar phosphomimetic and non-phosphorylatable mutants in the $InsP_3R$-2.

Significant progress has been made in probing the mechanism responsible for the effects of phosphorylation on the $InsP_3R$-1. Herein, it was noted that a potential mechanism for changing the apparent sensitivity of the $InsP_3R$ following phosphorylation could be

through alteration of the allosteric regulation of the receptor by ATP. Interestingly, it is also known that a unique nucleotide binding motif was created by the removal of 40 amino acids in the S2- (non-neuronal) splice variant of the $InsP_3R$-1 which was not present in the S2+ (neuronal) $InsP_3R$-1 (see Fig 27A). A mutation in this nucleotide binding site was created by altering a critical glycine residue to alanine. As shown in Fig. 27B, when this construct was expressed in DT-403ko cells the signature PKA-dependent potentiation of the carbachol-induced Ca^{2+} elevation was strikingly absent (compare with Fig. 25A). However, a full concentration-response curve (generated in the same fashion to that illustrated in Fig. 18) demonstrated that this construct was still capable of producing a maximal response comparable to that seen in the wild-type receptor – only the sensitivity to phosphorylation was altered. The absence of PKA-mediated potentiation occurs because this construct was now not susceptible to phosphorylation in the absence of ATP binding as shown in figure 28. These data also indicate that the degree of binding of ATP, possibly reflecting the metabolic status of the cell, can tune the sensitivity of the receptor to the effects of PKA phosphorylation.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains. The references disclosed are also individually and specifically incorporated by reference herein for the material contained in them that is discussed in the sentence in which the reference is relied upon.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

REFERENCES

1. Berridge, M.J. (1993) Nature. 361(6410), 315-25
2. Furuichi, T., S. Yoshikawa, A. Miyawaki, K. Wada, N. Maeda, and K. Mikoshiba. (1989) Nature. 342(6245), 32-8
3. Furuichi, T., S. Yoshikawa, and K. Mikoshiba. (1989) Nucleic Acids Res. 17(13), 5385-6

4. Sudhof, T.C., C.L. Newton, B.T. Archer, 3rd, Y.A. Ushkaryov, and G.A. Mignery. (1991) Embo J. 10(11), 3199-206

5. Maranto, A.R. (1994) J Biol Chem. 269(2), 1222-30

6. Nakagawa, T., H. Okano, T. Furuichi, J. Aruga, and K. Mikoshiba. (1991) Proc Natl Acad Sci U S A. 88(14), 6244-8

7. Danoff, S.K., C.D. Ferris, C. Donath, G.A. Fischer, S. Munemitsu, A. Ullrich, S.H. Snyder, and C.A. Ross. (1991) Proc Natl Acad Sci U S A. 88(7), 2951-5

8. Yoshikawa, F., M. Morita, T. Monkawa, T. Michikawa, T. Furuichi, and K. Mikoshiba. (1996) J Biol Chem. 271(30), 18277-84

9. Mignery, G.A. and T.C. Sudhof. (1990) Embo J. 9(12), 3893-8

10. Michikawa, T., H. Hamanaka, H. Otsu, A. Yamamoto, A. Miyawaki, T. Furuichi, Y. Tashiro, and K. Mikoshiba. (1994) J Biol Chem. 269(12), 9184-9

11. Boehning, D., D.O. Mak, J.K. Foskett, and S.K. Joseph. (2001) J Biol Chem. 276(17), 13509-12

12. Patel, S., S.K. Joseph, and A.P. Thomas. (1999) Cell Calcium. 25(3), 247-64

13. Berridge, M.J., P. Lipp, and M.D. Bootman. (2000) Nat Rev Mol Cell Biol. 1(1), 11-21

14. Bootman, M.D., T.J. Collins, C.M. Peppiatt, L.S. Prothero, L. MacKenzie, P. De Smet, M. Travers, S.C. Tovey, J.T. Seo, M.J. Berridge, F. Ciccolini, and P. Lipp. (2001) Semin Cell Dev Biol. 12(1), 3-10

15. Mignery, G.A., P.A. Johnston, and T.C. Sudhof. (1992) J Biol Chem. 267(11), 7450-5

16. Bezprozvanny, I., J. Watras, and B.E. Ehrlich. (1991) Nature. 351(6329), 751-4

17. Iino, M. (1990) J Gen Physiol. 95(6), 1103-22

18. Iino, M. (1991) J Gen Physiol. 98(4), 681-98

19. Ehrlich, B.E. and J. Watras. (1988) Nature. 336(6199), 583-6

20. Yamada, M., A. Miyawaki, K. Saito, T. Nakajima, M. Yamamoto-Hino, Y. Ryo, T. Furuichi, and K. Mikoshiba. (1995) Biochem J. 308 (Pt 1), 83-8

21. Tertyshnikova, S., X. Yan, and A. Fein. (1998) J Physiol. 512 (Pt 1), 89-96

22. Tertyshnikova, S. and A. Fein. (1998) Proc Natl Acad Sci U S A. 95(4), 1613-7

23. Tang, T.S., H. Tu, Z. Wang, and I. Bezprozvanny. (2003) J Neurosci. 23(2), 403-15

24. Nakade, S., S.K. Rhee, H. Hamanaka, and K. Mikoshiba. (1994) J Biol Chem. 269(9), 6735-42

25. Supattapone, S., S.K. Danoff, A. Theibert, S.K. Joseph, J. Steiner, and S.H. Snyder. (1988) Proc Natl Acad Sci U S A. 85(22), 8747-50

26. Wojcikiewicz, R.J. and S.G. Luo. (1998) J Biol Chem. 273(10), 5670-7

27. Jayaraman, T., K. Ondrias, E. Ondriasova, and A.R. Marks. (1996) Science. 272(5267), 1492-4

28. Giovannucci, D.R., G.E. Groblewski, J. Sneyd, and D.I. Yule. (2000) J Biol Chem. 275(43), 33704-11

29. Bruce, J.I., T.J. Shuttleworth, D.R. Giovannucci, and D.I. Yule. (2002) J Biol Chem. 277(2), 1340-8

30. Joseph, S.K. and S.V. Ryan. (1993) J Biol Chem. 268(31), 23059-65

31. Ferris, C.D., R.L. Huganir, D.S. Bredt, A.M. Cameron, and S.H. Snyder. (1991) Proc Natl Acad Sci U S A. 88(6), 2232-5

32. den Dekker, E., J.W. Heemskerk, G. Gorter, H. van der Vuurst, J. Donath, C. Kroner, K. Mikoshiba, and J.W. Akkerman. (2002) Arterioscler Thromb Vasc Biol. 22(1), 179-86

33. DeSouza, N., S. Reiken, K. Ondrias, Y.M. Yang, S. Matkovich, and A.R. Marks. (2002) J Biol Chem. 277(42), 39397-400

34. Ferris, C.D., A.M. Cameron, D.S. Bredt, R.L. Huganir, and S.H. Snyder. (1991) Biochem Biophys Res Commun. 175(1), 192-8

35. Haug, L.S., V. Jensen, O. Hvalby, S.I. Walaas, and A.C. Ostvold. (1999) J Biol Chem. 274(11), 7467-73

36. Komalavilas, P. and T.M. Lincoln. (1994) J Biol Chem. 269(12), 8701-7

37. Komalavilas, P. and T.M. Lincoln. (1996) J Biol Chem. 271(36), 21933-8

38. LeBeau, A.P., D.I. Yule, G.E. Groblewski, and J. Sneyd. (1999) J Gen Physiol. 113(6), 851-72

39. Pieper, A.A., D.J. Brat, E. O'Hearn, D.K. Krug, A.I. Kaplin, K. Takahashi, J.H. Greenberg, D. Ginty, M.E. Molliver, and S.H. Snyder. (2001) Neuroscience. 102(2), 433-44

40. Koga, T., Y. Yoshida, J.Q. Cai, M.O. Islam, and S. Imai. (1994) J Biol Chem. 269(15), 11640-7

41. Straub, S.V., D.R. Giovannucci, J.I. Bruce, and D.I. Yule. (2002) J Biol Chem. 277(35), 31949-56

42. Miyakawa, T., A. Maeda, T. Yamazawa, K. Hirose, T. Kurosaki, and M. Iino. (1999) Embo J. 18(5), 1303-8

43. Sugawara, H., M. Kurosaki, M. Takata, and T. Kurosaki. (1997) Embo J. 16(11), 3078-88

44. Kubista, H., T. Hawkins, and S.E. Moss. (1998) Biochim Biophys Acta. 1448(2), 299-310

45. Li, W., J. Llopis, M. Whitney, G. Zlokarnik, and R.Y. Tsien. (1998) Nature. 392(6679), 936-41

46. Cavallini, L., M. Coassin, A. Borean, and A. Alexandre. (1996) J Biol Chem. 271(10), 5545-51

47. Wojcikiewicz, R.J. (1995) J Biol Chem. 270(19), 11678-83

48. Rapoport, R.M., M.B. Draznin, and F. Murad. (1982) Proc Natl Acad Sci U S A. 79(21), 6470-4

49. Colbran, J.L., S.H. Francis, A.B. Leach, M.K. Thomas, H. Jiang, L.M. McAllister, and J.D. Corbin. (1992) J Biol Chem. 267(14), 9589-94

50. Houslay, M.D. and G. Milligan. (1997) Trends Biochem Sci. 22(6), 217-24

51. Ammendola, A., A. Geiselhoringer, F. Hofmann, and J. Schlossmann. (2001) J Biol Chem. 276(26), 24153-9

52. Schlossmann, J., A. Ammendola, K. Ashman, X. Zong, A. Huber, G. Neubauer, G.X. Wang, H.D. Allescher, M. Korth, M. Wilm, F. Hofmann, and P. Ruth. (2000) Nature. 404(6774), 197-201

53. Nezu, A., et al., *Evidence that zymogen granules do not function as an intracellular Ca2+ store for the generation of the Ca2+ signal in rat parotid acinar cells.* Biochem J, 2002. 363(Pt 1): p. 59-66.

54. Hassoni, A.A. and P.T. Gray, *Flash photolysis studies of the localization of calcium release sites in rat parotid isolated acinar cells.* J Physiol, 1994. 478 Pt 3: p. 461-7.

55. Sneyd, J., et al., *A model of calcium waves in pancreatic and parotid acinar cells.* Biophys J, 2003. 85(3): p. 1392-405.

56. Bruce, J.I., S.V. Straub, and D.I. Yule, *Crosstalk between cAMP and Ca(2+) signaling in non-excitable cells.* Cell Calcium, 2003. 34(6): p. 431-44.

57. Bruce, J.I., D.I. Yule, and T.J. Shuttleworth, *Ca2+-dependent protein kinase--a modulation of the plasma membrane Ca2+-ATPase in parotid acinar cells.* J Biol Chem, 2002. 277(50): p. 48172-81.

58. Mertz, L.M., B.J. Baum, and I.S. Ambudkar, *Refill status of the agonist-sensitive Ca2+ pool regulates Mn2+ influx into parotid acini.* 1990. 265(25): p. 15010-15014.

59. Putney, J.W., Jr., *Biphasic modulation of potassium release in rat parotid gland by carbachol and phenylephrine.* 1976. 198(2): p. 375-384.

60. Mertz, L.M., et al., *Calcium entry in rat parotid acini: activation by carbachol and aluminum fluoride.* 1990. 258(4 Pt 1): p. C654-C661.

61. Turner, R.J., *Mechanisms of fluid secretion by salivary glands.* Ann N Y Acad Sci, 1993. 694: p. 24-35.

62. Mandel, I.D., *The role of saliva in maintaining oral homeostasis.* J Am Dent Assoc, 1989. 119(2): p. 298-304.

63. Atkinson, J.C. and P.C. Fox, *Salivary gland dysfunction.* Clin Geriatr Med, 1992. 8(3): p. 499-511.

64. Baum, B.J., *Principles of saliva secretion.* Ann N Y Acad Sci, 1993. 694: p. 17-23.

65. Petersen, O.H., *Stimulus-secretion coupling: cytoplasmic calcium signals and the control of ion channels in exocrine acinar cells.* J Physiol, 1992. 448: p. 1-51.

66. Frizzell, R.A., M. Field, and S.G. Schultz, *Sodium-coupled chloride transport by epithelial tissues.* Am J Physiol, 1979. 236(1): p. F1-8.

67. Melvin, J.E., *Chloride channels and salivary gland function.* Crit Rev Oral Biol Med, 1999. 10(2): p. 199-209.

68. Nauntofte, B., *Regulation of electrolyte and fluid secretion in salivary acinar cells.* Am J Physiol, 1992. 263(6 Pt 1): p. G823-37.

69. Evans, R.L., et al., *Severe impairment of salivation in Na+/K+/2Cl- cotransporter (NKCC1)-deficient mice.* J Biol Chem, 2000. 275(35): p. 26720-6.

70. Melvin, J.E. and R.J. Turner, *Cl- fluxes related to fluid secretion by the rat parotid: involvement of Cl(-)-HCO3- exchange.* Am J Physiol, 1992. 262(3 Pt 1): p. G393-8.

71. Turner, R.J. and J.N. George, *Cl(-)-HCO3- exchange is present with Na+-K+-Cl- cotransport in rabbit parotid acinar basolateral membranes.* Am J Physiol, 1988. 254(3 Pt 1): p. C391-6.

72. Turner, R.J., J.N. George, and B.J. Baum, *Evidence for a Na+/K+/Cl- cotransport system in basolateral membrane vesicles from the rabbit parotid.* J Membr Biol, 1986. 94(2): p. 143-52.

73. Garrett, J.R. and A. Kidd, *The innervation of salivary glands as revealed by morphological methods.* Microsc Res Tech, 1993. 26(1): p. 75-91.

74. Tojyo, Y., A. Tanimura, and Y. Matsumoto, *Imaging of intracellular Ca2+ waves induced by muscarinic receptor stimulation in rat parotid acinar cells.* Cell Calcium, 1997. 22(6): p. 455-62.

75. Watson, E.L., et al., *Identification of muscarinic receptor subtypes in mouse parotid gland.* Am J Physiol, 1996. 271(3 Pt 1): p. C905-13.

76. Watson, E.L., J.A. Williams, and I.A. Siegel, *Calcium mediation of cholinergic-stimulated amylase release from mouse parotid gland.* Am J Physiol, 1979. 236(5): p. C233-7.

77. Sawaki, K., et al., *Involvement of G alpha q/11 in m3-muscarinic receptor stimulation of phosphatidylinositol 4,5 bisphosphate-specific phospholipase C in rat parotid gland membranes.* Arch Biochem Biophys, 1993. 305(2): p. 546-50.

78. Evans, R.L. and R.J. Turner, *Upregulation of Na(+)-K(+)-2Cl- cotransporter activity in rat parotid acinar cells by muscarinic stimulation.* J Physiol, 1997. 499 (Pt 2): p. 351-9.

79. Ambudkar, I.S., *Regulation of calcium in salivary gland secretion.* 2000. 11(1): p. 4-25.

80. Giovannucci, D.R., et al., *Cytosolic Ca(2+) and Ca(2+)-activated Cl(-) current dynamics: insights from two functionally distinct mouse exocrine cells.* J Physiol, 2002. 540(Pt 2): p. 469-84.

81. Zhang, X., et al., *Ryanodine and inositol trisphosphate receptors are differentially distributed and expressed in rat parotid gland.* Biochem J, 1999. 340 (Pt 2): p. 519-27.

82. Lee, M.G., et al., *Polarized expression of Ca2+ channels in pancreatic and salivary gland cells. Correlation with initiation and propagation of [Ca2+]i waves.* J Biol Chem, 1997. 272(25): p. 15765-70.

83. Tanimura, A., et al., *Isoproterenol potentiates alpha-adrenergic and muscarinic receptor-mediated Ca2+ response in rat parotid cells.* Am J Physiol, 1999. 276(6 Pt 1): p. C1282-7.

84. Tanimura, A., Y. Matsumoto, and Y. Tojyo, *Evidence that isoproterenol-induced Ca2(+)-mobilization in rat parotid acinar cells is not mediated by activation of beta-adrenoceptors.* Biochim Biophys Acta, 1990. 1055(3): p. 273-7.

85. Mak, D.O. and J.K. Foskett, *Single-channel inositol 1,4,5-trisphosphate receptor currents revealed by patch clamp of isolated Xenopus oocyte nuclei.* J Biol Chem, 1994. 269(47): p. 29375-8.

86. Mak, D.O., S. McBride, and J.K. Foskett, *ATP regulation of type 1 inositol 1,4,5-trisphosphate receptor channel gating by allosteric tuning of Ca(2+) activation.* J Biol Chem, 1999. 274(32): p. 22231-7.

87. Mak, D.O., et al., *Single-channel properties in endoplasmic reticulum membrane of recombinant type 3 inositol trisphosphate receptor.* J Gen Physiol, 2000. 115(3): p. 241-56.

88. Boehning, D., et al., *Single-channel recordings of recombinant inositol trisphosphate receptors in mammalian nuclear envelope.* Biophys J, 2001. 81(1): p. 117-24.

89. Mak, D.O., S. McBride, and J.K. Foskett, *Regulation by Ca2+ and inositol 1,4,5-trisphosphate (InsP3) of single recombinant type 3 InsP3 receptor channels. Ca2+ activation uniquely distinguishes types 1 and 3 insp3 receptors.* J Gen Physiol, 2001. 117(5): p. 435-46.

90. Nakagawa, T., Shiota, C., Okano, H., and Mikoshiba, K. (1991) *J. Neurochem.* 57, 1807–1810

91. Taylor, C. W., Genazzani, A. A., and Morris, S. A. (1999) *Cell Calcium* 26, 237–251

92. Joseph, S. K., Boehning, D., Pierson, S., and Nicchitta, C. V. (1997) *J. Biol.Chem.* 272, 1579–1588

93. Galvan, D. L., Borrego-Diaz, E., Perez, P. J., and Mignery, G. A. (1999) *J. Biol. Chem.* 274, 29483–29492

94. Yoshikawa, F., Iwasaki, H., Michikawa, T., Furuichi, T., and Mikoshiba, K. (1999) *J. Biol. Chem.* 274, 328–334

95. Ramos-Franco, J., Galvan, D., Mignery, G. A., and Fill, M. (1999) *J. Gen. Physiol.* 114, 243–250

96. Galvan, D. L., and Mignery, G. A. (2002) *J. Biol. Chem.* 277, 48248–48260 16. Parker, A. K., Gergely, F. V., and Taylor, C. W. (2004) *J. Biol. Chem.* 279, 23797–23805

97. Parker, A. K., Gergely, F. V., and Taylor, C. W. (2004) *J. Biol. Chem.* 279, 23797–23805

98. Taylor, C. W., and Laude, A. J. (2002) *Cell Calcium* 32, 321–334

99. Soulsby, M. D., Alzayady, K., Xu, Q., and Wojcikiewicz, R. J. (2004) *FEBS Lett.* 557, 181–184

100. Wagner, L. E., II, Li, W. H., and Yule, D. I. (2003) *J. Biol. Chem.* 278, 45811–45817

101. Kennelly, P. J., and Krebs, E. G. (1991) *J. Biol. Chem.* 266, 15555–15558

102. Tateyama, M., Rivolta, I., Clancy, C. E., and Kass, R. S. (2003) *J. Biol. Chem.*278, 46718–46726

103. Reiken, S., Lacampagne, A., Zhou, H., Kherani, A., Lehnart, S. E., Ward, C., Huang, F., Gaburjakova, M., Gaburjakova, J., Rosemblit, N., Warren, M. S., He, K. L., Yi, G. H., Wang, J., Burkhoff, D., Vassort, G., and Marks, A. R. (2003) *J. Cell Biol.* 160, 919–928

104. Tu, H., Miyakawa, T., Wang, Z., Glouchankova, L., Iino, M., and Bezprozvanny, I. (2002) *Biophys. J.* 82, 1995–2004

105. Boehning, D., and Joseph, S. K. (2000) *J. Biol. Chem.* 275, 21492–21499

106. Lin, C., Widjaja, J., and Joseph, S. K. (2000) *J. Biol. Chem.* 275, 2305–2311

107. Tu, H., Tang, T. S., Wang, Z., and Bezprozvanny, I. (2004) *J. Biol. Chem.* 279, 19375–19382

108. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) *Nat. Rev. Mol. Cell Biol.* 1, 11–21

109. Sneyd, J., Tsaneva-Atanasova, K., Yule, D. I., Thompson, J. L., and Shuttleworth, T. J. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 101, 1392–1396

110. Chatton, J. Y., Cao, Y., Liu, H., and Stucki, J. W. (1998) *Biochem. J.* 330, 1411–1416

111. Hajnoczky, G., Gao, E., Nomura, T., Hoek, J. B., and Thomas, A. P. (1993) *Biochem. J.* 293, 413–422

112. Joseph, S. K., and Ryan, S. V. (1993) *J. Biol. Chem.* 268, 23059–23065

113. Ando, H., Mizutani, A., Matsu-ura, T., and Mikoshiba, K. (2003) *J. Biol. Chem.* 278, 10602–10612

114. Boehning, D., Patterson, R. L., Sedaghat, L., Glebova, N. O., Kurosaki, T., and Snyder, S. H. (2003) *Nat. Cell Biol.* 5, 1051–1061

115. Maximov, A., Tang, T. S., and Bezprozvanny, I. (2003) *Mol. Cell. Neurosci.* 22, 271–283